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THE PHYSIOLOGY OF HOST–PARASITE RELATIONS

V. A PRELIMINARY EXAMINATION OF THE LEVEL OF FREE ENDOGENOUS INDOLEACETIC ACID IN RUSTED AND MILDEWED CEREAL LEAVES AND THEIR ABILITY TO DECARBOXYLATE EXOGENOUSLY SUPPLIED RADIOACTIVE INDOLEACETIC ACID[1]

MICHAEL SHAW AND A. R. HAWKINS[2]

Abstract

The growth substances were extracted with cold alcohol from the first leaves of uninfected, rusted (wheat), and mildewed (barley) cereal seedlings. The acid ether fractions were chromatographed on paper and the chromatograms were cut into sections which were assayed for growth promoting or inhibiting activity in the *Avena* coleoptile straight growth test. The estimated, free, endogenous indoleacetic acid content of uninfected leaves ranged from 0.5 to 3.2 μg. per kilogram fresh weight. In the early stages of infection this decreased, but increased again to from 5 to about 10 μg. per kilogram fresh weight by the 10th day after the inoculation of susceptible hosts. Indoleacetic acid was not detected in ungerminated uredospores of stem rust (race 15B), but two other growth promoting substances appeared to be present.

Leaf disks were incubated with radioactive indoleacetic acid (as $-C^{14}OOK$) and the radioactivity released as $C^{14}O_2$ was measured. The ability of the tissue to decarboxylate the indoleacetate ('oxidase' activity) increased sharply, sometimes to as much as 1000%, in the first 3 days after inoculation. With susceptible hosts, this increase was followed by an almost equally sharp decrease to less than 50% of the values for uninfected tissue. With infected, resistant tissue, the secondary decrease in 'oxidase' activity was delayed and less pronounced.

The results are discussed and a working hypothesis suggested with respect to the relation between susceptibility or resistance and the auxin balance.

Introduction

Indoleacetic acid is the best known of the native plant growth substances. In 1935 it was identified in the culture filtrate of *Rhizopus* (57) and it is now known to be produced by other fungi (7, 8, 26, 66, 67, 40). Yet, supplied exogenously, indoleacetic acid more often inhibits than stimulates fungal growth (26). Gentile and Klein (26) suggested that this was the result of the production of optimal amounts of indoleacetic acid by the organisms, so that additional amounts, supplied exogenously, raised the total concentration to an inhibitory level. They showed that *Diplodia* produced indoleacetic acid, that its growth was inhibited by trichlorophenoxyacetic acid, and that the effect of the latter was reversed by indoleacetic acid.

[1]Manuscript received June 24, 1957.
Contribution from the Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan.
[2]Graduate student.

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The inhibiting effects on fungi of small amounts of indoleacetic acid led Défago to suggest in 1941 (13) that auxin might play a role in conferring resistance to fungal parasites. This appears to be the first suggestion of a relationship between indoleacetic acid and disease resistance.

Since many fungi produce indoleacetic acid and therefore probably also indoleacetic acid oxidase (see 26), it is scarcely surprising that many plant pathogens, including members of the Pucciniales and Erysiphales, cause morphological and therefore physiological changes in their hosts which suggest an interference with the metabolism of growth substances (1). For example, only those species of *Ustilago* (66) and *Nectria* (7) which produce indoleacetic acid *in vitro* cause gall formation on their hosts. An increased indoleacetic acid content is also associated with gall formation by *Agrobacterium tumefaciens* (37). On the other hand, *Omphalia flavida* apparently secretes an oxidase which reduces the auxin content in the leaves of the coffee plant, causing their premature abscission (52). Infection with *Erysiphe polygoni* also causes a premature leaf abscission in beans (42).

Pilet (44, 45) found that the auxin contents of leaves of *Sempervivum* infected with *Endophyllum sempervivi* and of *Euphorbia* infected with *Uromyces pisi* were increased 100- and 1000-fold, respectively. Marked morphological and physiological disturbances were associated with these apparently enhanced auxin levels. Pilet's (44, 45) estimates of auxin contents were based solely on the effect of non-chromatographed chloroform extracts in the *Avena* coleoptile curvature test. Hirata (34) used juice pressed from the tissue or the diffusate from pieces of tissue in this test. He reported 1.6-, 5.0-, and 8.4-fold increases in auxin in aecial infections of *Gymnosporangium* on *Pyrus*, of *Puccinia* on *Smilax*, and of *Uromyces* on *Ranunculus* respectively. It is important to note that Hirata (34) also found increased *Avena* curvatures with tissues infected with a number of facultative parasites. Virus-infected tissue of sweet potato gave reduced *Avena* curvatures. Hirata (34) concluded that the abnormal growth (hypertrophy or dwarfing) caused by the fungi or virus was correlated proportionately with the amount of free auxin in the infected tissues. However, neither Hirata (34) nor Pilet (44, 45) specifically identified indoleacetic acid in the infected tissues.

Rusted and mildewed wheat and barley leaves do not show any obvious morphological abnormalities. Unlike maize and many dicotyledons, these cereals do not show marked hypertrophic or hyperplasic responses to exogenously applied indoleacetic acid, nor, it may be noted, has the successful tissue culture of cereal leaf tissue been reported. It was therefore considered of importance to examine the indoleacetic acid content and oxidase activity of rusted and mildewed wheat and barley leaves. The results of a preliminary examination, started by the senior author in the summer of 1953, and a hypothesis regarding the role of auxin in the host–parasite relationship are presented in this paper.

Plant Material

Little Club (rust reaction type 4) and Khapli (rust reaction type 1) wheats and Atlas (mildew reaction type 4) and Gatami (mildew reaction type 1) barleys were grown and heavily inoculated with race 15B of stem rust and race 3 of powdery mildew, respectively. The growing conditions and methods of inoculation have already been described (49, 53).

Methods

Extraction, Separation, and Assay of Growth Substances

The plant material was frozen over solid carbon dioxide and the growth substances extracted with cold ethanol. The 'acid' ether-soluble fractions of the extracts were chromatographed on Whatman No. 1 paper using isopropanol: water in the proportions of 10:1 in an atmosphere containing ammonia. The completed chromatograms were assayed by the *Avena* coleoptile straight growth test. The methods used were closely similar to those described in (5) and (36).

The chromatography of the growth substances and the production of *Avena* coleoptiles were so scheduled that it was never necessary to store the chromatograms. As soon as these were dry they were cut into 10 equal sections, numbered 1 to 10 from the origin to the front. Each section was placed in a syracuse watch glass with 2 ml. of distilled water and 10 *Avena* coleoptile sections (5 mm. long) were floated on the water. This operation was performed under weak red light and the dishes were placed in darkness at 21 $\pm$ 1° C. for 20–24 hours, with occasional gentle shaking during the first few hours. Extracts of infected and uninfected tissue were chromatographed and assayed simultaneously. Coleoptile growth in distilled water and in a series of concentrations of indoleacetic acid was always determined at the same time in order to provide data for estimation of the indoleacetic acid concentration of the tissue. The mean increase in length of the coleoptile sections on water for all occasions and both varieties of oats was 9.3% (s = 2.99). Points on the bio-assay curves (Figs. 1 to 5) have only been considered significant if they differ from the appropriate control values by more than 2.99%.

The *Avena* coleoptiles were grown and prepared as follows: hulled seeds of Victory or Brighton (hulless) oats were soaked in water for 2–4 hours and the water was discarded. The seeds were planted, embryo side down, in wet Ottawa quartz sand in wooden boxes, and grown in continuous dim red light provided by a 7.5 watt bulb and a Corning 2404 filter (41) at 21 $\pm$ 1° C. until the coleoptiles were 20–30 mm. long. Working in dim red light and using a special cutter, the tip (3 mm.) of each coleoptile was discarded and the next 5 mm. retained. The young leaves were not removed from the coleoptiles. According to Bentley and Housley (6) they do not interfere with the assay.

Indoleacetic Acid Destruction

One hundred leaf disks (2.8 mm. diameter) were incubated for 3 hours in 3 ml. of 0.05 *M* KH_2PO_4 containing 50 μg. of the potassium salt of radioactive indoleacetic acid ($-C^{14}OOK$; specific activity = 0.70 microcuries per mg.) in Warburg flasks at 25 ± 0.02° C. The radioactive carbon dioxide released by decarboxylation of the indoleacetate was absorbed in potassium hydroxide in the center wells of the flasks, collected at the end of the incubation period, and finally converted to barium carbonate for measurements of radioactivity. These operations were carried out as described for radioactive glucose in Part III (54). 'Standard' barium carbonate plaques containing the radioactivity released by complete decarboxylation of 50 μg. of the potassium indoleacetate gave 21,000 ± 2% c.p.m. The decarboxylation of indoleacetic acid approximately parallels its oxidation by plant extracts (39). Decarboxylation by boiled tissue was less than 10% of that by unboiled tissue and, in preliminary experiments, the enzymic decarboxylation was found to be independent of the area of cut surface. Since 100 leaf disks were always used, the area of cut surface was virtually constant in all experiments. Galston and Dahlberg (20) have carried out *in vivo* assays for indoleacetic acid oxidase by measuring the disappearance of indoleacetic acid from the medium caused by known weights of tissue (pea epicotyl). In experiments with both uninfected and rusted tissue Miss Ann Oaks has found that the decarboxylation of indoleacetic acid after 2 hours is approximately 85% of that after 3 hours' incubation. In this paper the radioactivity evolved as carbon dioxide during an incubation period of 3 hours is taken as an approximate measure of the ability of the tissue to decarboxylate indoleacetic acid.

Results

Growth Substance Assays

Figs. 1 and 2 show the distribution of growth substances, active in the *Avena* straight growth test, which are present in the acid fractions of alcohol extracts of uninfected and mildewed first leaves of Atlas barley at 5 and 10 days after inoculation. At 5 days after inoculation the indoleacetic acid content of mildewed leaves was less than one-fifth of that of uninfected leaves (Fig. 1, Table I). Five days later the indoleacetic acid content of the mildewed leaves was approximately equal to that of the uninfected leaves (Fig. 2, Table I). In another experiment (Table I) 90 g. lots of mildewed and uninfected leaves were extracted and chromatographed. When sprayed with 1% paradimethylaminobenzaldehyde the chromatogram for the mildewed leaves developed a single spot of the same color, stability, and R_f as a 'marker' spot of indoleacetic acid. From the size of the spot and the spots formed using known amounts of indoleacetic acid, the indoleacetic acid content of the mildewed leaves was estimated to be between 5 and 10 μg. per kilogram fresh weight (Table I). There was not enough indoleacetic acid on the

chromatogram for the uninfected leaves to give a visible spot with the reagent. It is therefore certain that the indoleacetic acid content of the uninfected leaves could not have exceeded 1–2 μg. per kilogram fresh weight.

TABLE I

INDOLEACETIC ACID CONCENTRATIONS IN UNINFECTED, RUSTED WHEAT AND MILDEWED BARLEY LEAVES

Material	Days after		IAA (μg./kg. fresh wt.)		See figure
	Planting	Inoc.	Uninfected	Infected	
Atlas barley	11	5	2.3	Not detected (<0.5)	1
	18	10	3.2	3.5	2
	18	10	Not >1–2*	5–10*	–
Khapli wheat	14	5	0.5?	<0.5?	3
Little Club wheat	22	11	0.5	12.0	4

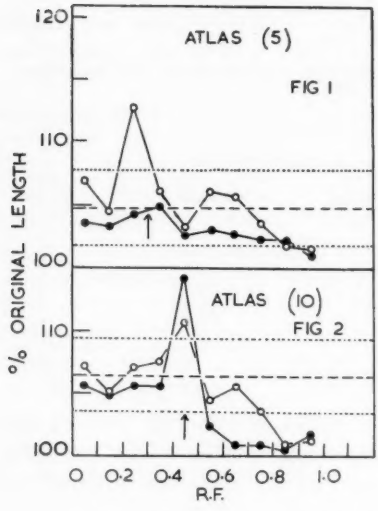
*Chromatograms sprayed with *p*-dimethylaminobenzaldehyde.

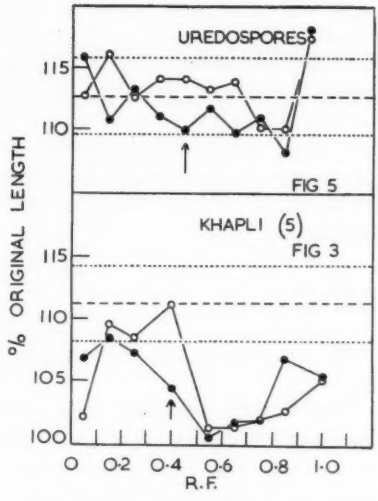
Growth inhibiting substances with R_f values of 0.55 and 0.75–1.0 also appeared to be present in uninfected barley leaves (Figs. 1 and 2). With the extracts of mildewed leaves, there was a general inhibition of growth at R_f values greater than that of indoleacetic acid (Figs. 1 and 2).

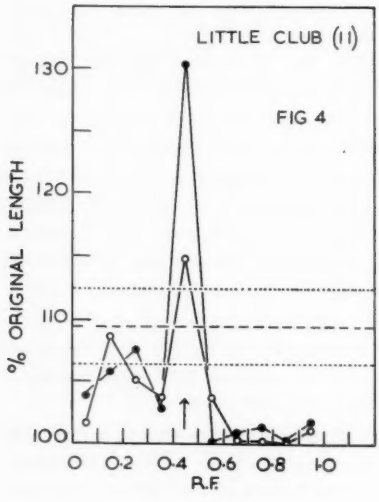
The distribution of growth substances in the extracts of rusted and uninfected first leaves of Khapli and Little Club wheats are shown in Figs. 3 and 4. Unfortunately, sections Nos. 4 and 5 of the chromatograms for Khapli were assayed together. If this is taken into account, it will be seen that the general pattern of the graph for uninfected Khapli leaves is similar to that for uninfected leaves of Little Club. On the other hand, there was a definite inhibition of growth at the locus of indoleacetic acid in infected leaves of Khapli. It is thus probable that, at 5 days after inoculation, rust-infected leaves of Khapli contained less indoleacetic acid than did uninfected leaves. It is at once clear from Fig. 4 that, at 10 days after inoculation, rust-infected leaves of Little Club contained much more indoleacetic acid than uninfected leaves (Table I).

Figs. 3 and 4 also suggest the presence of two growth inhibiting substances with R_f values lower than indoleacetic acid. In addition there was a general inhibition of growth at R_f values above that of indoleacetic acid.

Fig. 5 illustrates the distribution of growth substances observed in extracts of ungerminated uredospores of stem rust (mainly Race 15B). The most striking feature of these graphs is the apparent absence of any definite activity which can be attributed to indoleacetic acid, and the presence of at least one and possibly two other growth stimulating substances, not found in the leaf extracts, and having R_f values of about 0.10 and 0.95, respectively. The indoleacetic acid equivalents of these spots are given in Table II. Kefford


ATLAS (5)
FIG 1
ATLAS (10)
FIG 2
% ORIGINAL LENGTH
R.F.


UREDOSPORES
FIG 5
KHAPLI (5)
FIG 3
% ORIGINAL LENGTH
R.F.


LITTLE CLUB (11)
FIG 4
% ORIGINAL LENGTH
R.F.

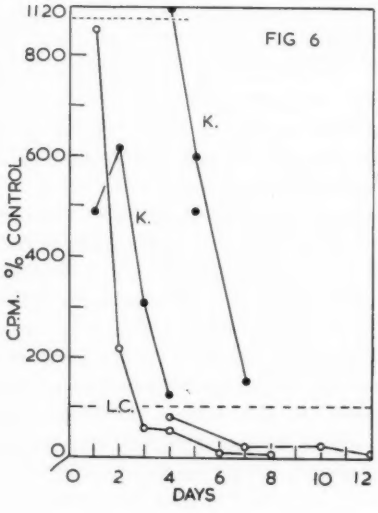


FIG 6
K.
K.
L.C.
C.P.M. % CONTROL
DAYS

(36) has shown that accelerator α, inhibitor β, and a substance provisionally identified as indoleacetonitrile are of widespread occurrence in plant tissues. The possibility that the two growth promoting substances in the uredospores are, respectively, accelerator α (R_f 0.10) and indoleacetonitrile (R_f 0.95) should be borne in mind. Inhibitor β may also account for some of the inhibitions observed at R_f values greater than that of indoleacetic acid on the chromatograms of the leaf extracts.

TABLE II

THE INDOLEACETIC ACID 'EQUIVALENTS' OF THE GROWTH PROMOTING SUBSTANCES IN UREDOSPORES OF STEM RUST (RACE 15B)

	IAA equivalent (μg. IAA/kg. fresh weight)	
	R_f = 0.1	R_f = 0.95
6 g. uredospores extracted whole	6	9
2.5 g. uredospores ground in ball mill	20	30

The dry weight of uredospores is approximately 90% of the fresh weight.

From the graphs in Figs. 1, 2, 3, 4, and 5 and 'calibration' curves for the elongation of *Avena* coleoptiles in indoleacetic acid, it was possible to estimate the approximate concentrations of indoleacetic acid, or its equivalent, in the plant materials. These estimates are given in Tables I and II. Since the calibration curves were prepared using standard, non-chromatographed solutions of indoleacetic acid, no allowance has been made for possible losses during chromatography. The methods employed are believed to have kept these to a minimum level. The estimates in Table I are in general agreement with published figures for other plant tissues, some of which are given in

FIGS. 1 to 5. *Avena* coleoptile extension vs. R_f for paper partition chromatograms of the acid ether fractions of alcohol extracts of plant material (fresh weights). Arrows indicate R_f values of 'marker' spots of indoleacetic acid. Symbols: ○ uninfected leaves; ● infected leaves (except in Fig. 5).

FIG. 1. 26 g. uninfected and 26 g. mildewed first leaves of Atlas barley. Five days after inoculation.

FIG. 2. 15 g. uninfected and 19 g. mildewed first leaves of Atlas barley. Ten days after inoculation.

FIG. 3. 99 g. uninfected and 99 g. rusted first leaves of Khapli wheat. Five days after inoculation.

FIG. 4. 80 g. uninfected and 81.5 g. rusted first leaves of Little Club wheat. Eleven days after inoculation.

FIG. 5. Open circles, 6.0 g. uredospores of stem rust, extracted whole. Closed circles, 2.5 g. uredospores, ground in ball mill.

FIG. 6. Decarboxylation of radioactive indoleacetic acid by rust infected first leaves of Little Club and Khapli wheats. Ordinate: c.p.m. for infected as % of value for uninfected from data of Table IV. Abscissa: days after inoculation.

Table III. Nevertheless, in considering the results, certain limitations of the method of assay should be borne in mind. Because of its small volume (2 ml.) the concentration of the ambient solution may have declined appreciably as a result of absorption by the coleoptile sections. On this account the estimated indoleacetic acid levels may be too low. It is also possible that those substances which caused an inhibition of growth in the standard 20–24 hour assay might stimulate growth over shorter time periods. In this connection, serial dilutions of the substances in each section of each chromatogram should be subjected to the *Avena* straight growth assay.

TABLE III

FREE AUXIN CONCENTRATIONS IN LEAVES, SHOOTS, AND ROOTS

Species	Organ	IAA (or equivalent) (μg./kg. fresh wt.)	Reference and method of assay
Pineapple	Young leaves	0.5–0.8	Van Overbeek *et al.* (53). *Avena* curvature assay of non-chromatographed extracts
	Semimature leaves	0.3–0.5	
	Mature leaves	0.4–0.7	
	Apex of axis	6.1	
Bean	Leaf blades		Shoji *et al.* (49). *Avena* curvature assay of non-chromatographed extracts
	(15 days old)	0.8	
	(30–60 days old)	2.2	
	Leaf stalks		
	(15 days old)	4	
	(30–60 days old)	0.5	
Broad bean	Whole seedlings (17–27 days old)	0.72 (n = 3)*	Audus and Thresh (2). Pea root segment assay of indoleacetic acid section of chromatograms
Pea	Roots (7–10 days old)	1.29 (n = 3)*	
Sunflower	Shoots (14–18 days old)	5.96 (n = 6)*	
Cabbage	Leaves	48.7 (n = 2)*	
Wintex barley	Leaves	Not detected† in 1 kg. fresh weight	Vlitos and Meudt (54). Chromatogram sprayed with paradimethyaminobenzaldehyde

*Mean values. n = number of analyses.
†Indicates indoleacetic acid content too low for detection by chromophore.

Indoleacetic Acid 'Oxidase'

The effect of the progressive development of rust and mildew on the decarboxylation of indoleacetic acid by leaf disks is illustrated in Figs. 6 and 7. The primary data are given in Table IV. Idealized curves are shown in Fig. 8. With both rusted and mildewed tissue, the extent of phase I (Fig. 8), during which indoleacetic acid is rapidly destroyed, was greater with the tissue of resistant hosts than with that of susceptible hosts.

TABLE IV

THE DESTRUCTION OF INDOLEACETIC ACID (—$C^{14}OOK$) BY UNINFECTED, RUSTED, AND MILDEWED LEAVES

Date	Days after		Activity of CO_2 evolved* (c.p.m. per 10 mg. dry wt. of tissue)	
	Planting	Inoc.	Uninfected	Infected
		Little Club Wheat		
Mar. 27/55	14	4	2145	1688
Feb. 29/55	15	7	6632	1313
Aug. 29/55	20	10	9196	2147
Aug. 31/55	22	12	5027	240
Dec. 6/55	21	13	13178	368
May 15/56	11	1	1413	11937
May 16/56	12	2	2339	5116
May 17/56	13	3	2207	1210
May 18/56	14	4	1995	1068
May 20/56	16	6	4934	387
May 22/56	18	8	2531	179
		Khapli Wheat		
Jan. 23/56	13	4	517	5764
Dec. 13/56	14	5	1353	6673
Dec. 15/56	16	7	4802	7271
Aug. 28/56	11	1	1284	6336
Aug. 29/56	12	2	1953	12070
Aug. 30/56	13	3	1185	3687
Aug. 31/56	14	4	3190	3913
Apr. 25/56	12	5	458	2715
		Atlas Barley		
Nov. 28/55	13	3	11467	15272
Feb. 28/55	14	4	3358	683
Oct. 25/55	12	6	15218	4483
Aug. 29/55†	12	7	10147	5207
Aug. 31/55	14	9	6915	2981
Oct. 29/55	16	10	3072	1433
		Gatami Barley		
Jan. 17/56	14	4	11215	23287
Jan. 18/56	15	5	9633	8535

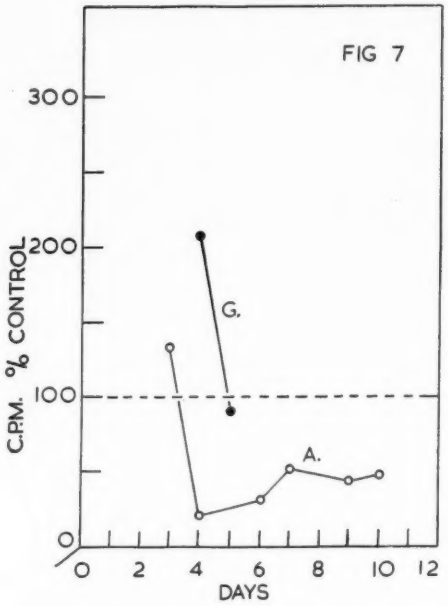
NOTE: Little Club and Khapli wheats were infected with stem rust; Atlas and Gatami barleys were infected with mildew; 100 leaf disks (diam. 2.8 mm.) were incubated with 50 μg. of radioactive potassium indoleacetate in 0.05 M KH_2PO_4 for 3 hours, except in the August 1956 experiments with Khapli wheat in which the incubation period was reduced to 2 hours.

*Figures are means of two or three replicate determinations.

†Uninfected plants 13 days old.

Discussion

There is apparently little or no free extractable indoleacetic acid in ungerminated rust uredospores (Fig. 5). At least one and possibly two other growth stimulating substances are present and may be equivalent in growth promoting activity to rather large amounts of indoleacetic acid (Table II). Possibly one or both of these substances may be converted to indoleacetic acid during germination. It is of interest that Audus and Thresh (2) found



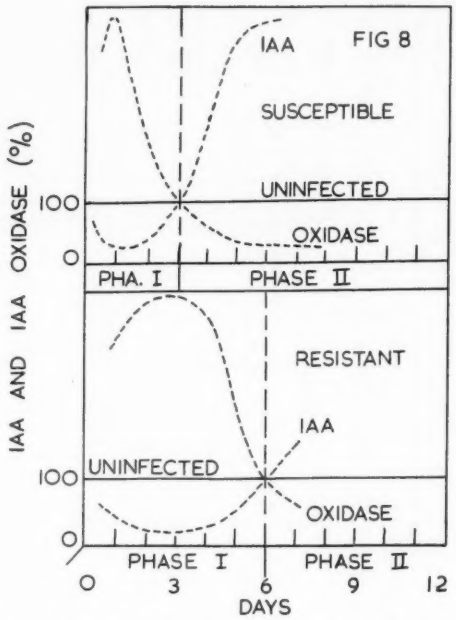


FIG. 7. Decarboxylation of radioactive indoleacetic acid by mildewed first leaves of Atlas and Gatami barleys. Ordinate and abscissa as for Fig. 6.

FIG. 8. Scheme to illustrate the relationship proposed between destruction of indoleacetic acid and the concentration of free endogenous indoleacetic acid in infected tissue of susceptible (top) and resistant (bottom) hosts. Ordinate: destruction of indoleacetic acid (oxidase) and indoleacetic acid content (IAA). Abscissa: days after inoculation. See discussion in text.

that sunflower seeds, even though soaked in water for 24 hours, contained no detectable free indoleacetic acid, which was, however, produced during germination.

On the other hand, indoleacetic acid appears to be the main growth substance both in the 'acid' fraction of alcohol extracts of uninfected wheat and barley leaves and in extracts of Little Club and Atlas leaves bearing well developed infections of rust and mildew, respectively. In the later stages of infection, the indoleacetic acid contents of rust-infected leaves of Little Club and mildew-infected leaves of Atlas were found to have increased by 10- to 20-fold and about 5-fold, respectively (Table I). More extensive work will be required to determine whether infection causes significant changes in other, mainly inhibiting, substances, which are active in the *Avena* straight growth test and which appear to be present in the leaves.

The increases in indoleacetic acid content found in the later stages of infection are too large to be explained by the increases in dry weight which accompany development of the parasites (49). They are of the same order of magnitude as the increases in auxin level suggested by Hirata (34) for the aecial infections of three different rusts but are much smaller than the increases (100- and 1000-fold) reported by Pilet (44, 45) for rusted leaves of *Euphorbia* and *Sempervivum*.

Wolf (67) has recently found that an isolate believed to be the cedar apple rust fungus (12) produces, on a medium containing tryptophan, amounts of indoleacetic acid corresponding to the increases in the auxin content of rusted tissue reported by Pilet (44, 45). Wolf has therefore suggested (67) that indoleacetic acid is produced by the fungus from tryptophan supplied by the host. In this laboratory, Colotelo (11) has found appreciable increases in the tryptophan content of wheat leaf pieces bearing well developed rust infections. While Wolf's work may be regarded as providing presumptive evidence that the excess indoleacetic acid is produced directly by the fungus, the possibility remains that the rates of production or destruction of indoleacetic acid in the host cells are also altered, as is apparently the case in crown gall tumor tissue (37). Baldacci (4) has pointed out that metabolic products of a parasite which were themselves inactive as growth promoting substances could affect auxin levels by influencing the metabolic pathways involved in the synthesis and degradation of auxin (see also 9).

Von Gutenberg and Beythien (62) consider that indoleacetic acid increases cell permeability. The increased indoleacetic acid content of infected, susceptible tissue and the decreased indoleacetic acid content of infected, resistant tissue might thus account for Thatcher's (56) finding that infection increases permeability when the host is susceptible and decreases it when the host is resistant.

It has been suggested that the activity of indoleacetic acid oxidase determines the endogenous level of auxin (20, 46). If the rate of destruction of indoleacetic acid, supplied exogenously to wheat leaf disks, reflects the rate of destruction of free endogenous indoleacetic acid and if this represents the

main control over the level of endogenous indoleacetic acid, it might be predicted from the graphs in Figs. 6 and 7 that the free indoleacetic acid content of rusted and mildewed wheat and barley leaves should fall below the level found in uninfected leaves during the first few days after inoculation and rise above this level in the later stages of infection, at least with susceptible hosts. With resistant hosts, such as Khapli, the initial phase of increased destruction of indoleacetic acid is apparently extended until degenerative changes develop and the secondary rise in indoleacetic acid content may not occur. Although scanty, the results of the bio-assays for indoleacetic acid (Figs. 1 to 5, Tables I and II) appear to be in reasonable agreement with this hypothesis (see Fig. 8).

Although the effect may be secondary, indoleacetic acid apparently promotes the translocation of nutrients to the locus of application (38) and Avery has suggested that the mobilization of substances at shoot apices and other growth centers may be influenced by their high auxin content (3). Rheinhold and Powell (47) have shown that indoleacetic acid stimulates the uptake of amino acids by sunflower hypocotyls. If indoleacetic acid is necessary for the mobilization of metabolites at the loci of infections and for the growth of the parasites, the duration of phase I (Fig. 8) during which indoleacetic acid is rapidly destroyed may well be a critical factor in determining the development of the parasite and the resistance or susceptibility of the host. It should be noted that the accumulation of radioactive sugars at rust infections on the susceptible species, Little Club, is not macroscopically detectable on autoradiographs of the leaves until the 5th or 6th day after inoculation (49); i.e. not until after the completion of phase I (Fig. 8).

Experimental treatments which shorten phase I (Fig. 8) and allow a faster transition to phase II (Fig. 8), during which the level of free endogenous indoleacetic acid rises, or which remove or inhibit competing or dominating centers, such as the apical meristem, might be expected to result in a partial or complete loss of resistance.

Treatments with DDT (14), maleic hydrazide (50), ionizing radiations (51), excesses of Zn^{++}, Mn^{++}, and Co^{++} (16), excision of leaves (50), and spraying with sucrose (48) all cause a loss of resistance to wheat stem rust, at least under some conditions. It should, however, be noted that Schwinghamer (51) found that ionizing radiations did not alter the resistance of flax to flax rust unless the rust itself was irradiated shortly after inoculation. Resistance to stem rust of wheat and oats and crown rusts of oats is often lowered by moderate increases in temperature (30, 35, 27, 63, 43, 31) and decreases in temperature may lower resistance to leaf rust (25, 31). Illumination also affects the expression of susceptibility to rust (15, 17, 35, 31), partly through its effects on the availability of carbohydrate. The 'mesothetic' type reaction of stem rust is particularly sensitive to light and temperature (35). Zscheile (68) has obtained preliminary evidence that light quality affects the expression of genes conferring resistance to bunt; the possibility that light quality affects rust development should not be ignored. Blue light is particularly effective

in increasing the rate of destruction of indoleacetic acid (19, 64, 65). Instances of 'seedling resistance' and 'mature plant susceptibility' are well known for leaf rust (10). Gassner and his associates (21, 22, 23, 24) have found in certain cases, that, in Chester's words (10) a 'wave of resistance' sweeps over the wheat plant before flowering, being both preceded and followed by susceptibility. This phenomenon was interpreted by Gassner as the result of successful competition of the developing inflorescences for metabolites produced in the leaves. Allen (1) has already drawn attention to the desirability of determining whether host susceptibility is influenced by the induction of flowering.

It is now thought that the level of free endogenous indoleacetic acid falls and the *in vivo* activity of indoleacetic acid oxidase rises as tissues age (20). Oxidation of indoleacetic acid is increased by illumination (18, 19, 28, 29, 64). Moreover, the activity of the oxidase appears to depend on a delicate balance between cationic (particularly Mn^{++}) and phenolic cofactors (33, 39). In view of these points and of the data presented in this paper, it does not seem unreasonable to suggest that factors which affect resistance may act directly or through their effects on auxin metabolism to alter the balance between the infection-loci and competing growth centers in other parts of the growing plant. In a word, it has been the burden of this paper and of Part IV (50) to suggest, as a working hypothesis, that, whatever else may be involved, successful obligate parasitism by the rust and mildew fungi requires the early establishment of a local 'field' of 'dominance'.

Some additional support for this hypothesis stems from the work of Dr. Franziska Turel, who found that the development of flax rust on flax leaves in tissue culture was markedly influenced by the growth of the leaf tissue (58). Where little or no callus growth occurred, the fungus sporulated abundantly; when callus growth was excessive, development of the fungal mycelium was sparse and soon suppressed. With intermediate callus growth the fungus produced an extensive vegetative mycelium. Dr. Turel considers that growth of the mycelium depends on the right equilibrium between the parasite and the host tissue. The latter must be vigorous enough to produce a fair amount of callus, but must not grow too abundantly (59).

It is impossible at this stage to determine whether the observed and proposed effects of infection on auxin balance are in fact primary or whether they follow some other as yet unknown effect or effects. With this proviso, it may be suggested that genetic control of resistance, at least where resistance can be broken by experimental manipulations, may in fact be genetic control of the internal factors (e.g. availability and balance of cofactors in the 'oxidase' system) which govern auxin balance. Clearly, observations on the effect of infection on the levels of indoleacetic acid and its oxidase should be extended. In addition, it now seems important to examine experimentally the effects of factors which alter resistance with reference to the hypothesis outlined above, whether or not this is ultimately substantiated.

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STUDIES OF TILLETIA CONTRAVERSA, THE CAUSE OF DWARF BUNT OF WINTER WHEAT[1]

R. J. BAYLIS

Abstract

The occurrence and distribution of dwarf bunt of winter wheat in Ontario since 1952 is noted. While the severity of the disease has not been great, indications are that it is present in most areas of the province where winter wheat is produced in quantity and thus is of concern particularly to growers of seed wheat.

The germination of *T. contraversa* was studied with a view to determining the prerequisites for infection, and the development of dwarf bunt. Chlamydospores incubated on water agar and on ordinary soil at controlled temperatures between 0° and 15° C. germinated only after incubation for 3 to 4 weeks at relatively low temperature, and required almost 8 weeks to reach a maximum of over 50% of the spores germinated.

The germination process to the end point of sporidium development was dependent on light and a temperature about 5° C. for maximum development. A slightly acid soil reaction favored spore germination, but soil moisture was a more critical factor.

In field tests, a correlation was also shown to exist between temperature, light, and soil moisture, and the severity of dwarf bunt infection resulting in winter wheat seeded on infested soil. In continuing tests, chlamydospores have survived 3 years on the surface of soil with very little loss of viability.

Introduction

Dwarf bunt was first reported on winter wheat in Ontario in 1952. The pronounced dwarfing of infected plants distinguishes the disease from common bunt of wheat. The causal organism was considered by some workers (22) to be only varietally distinct from the rough-spored species of common bunt (*Tilletia caries* (DC.) Tul.), but because of differences in morphology and life cycle (5, 6, 8), it is now usually regarded as a distinct species *T. contraversa* Kühn (14).

In the United States, where dwarf bunt was recognized about 1931, research on the disease was at first chiefly concerned with the production of resistant varieties of wheat. This approach was reasonably successful. Within recent years, however, more fundamental and widespread investigations have been undertaken to explain the processes involved in the germination of the chlamydospores and in the infection of the host. Some progress has been made in the study of the pathogen, but no practical method of control of the disease has yet been developed. Accordingly, investigations of the dwarf bunt fungus as it occurs in Ontario were begun at the Ottawa laboratory of the Botany and Plant Pathology Division.

Occurrence of Dwarf Bunt in Ontario

According to Conners (4) and Page (16) dwarf bunt in Ontario was first recognized in 1952. Since then, annual surveys and seed examinations have established the presence of the disease in 25 counties, principally in the south

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and southwestern parts of the province where most of the winter wheat crop is grown. The distribution of dwarf bunt in Ontario is shown in Fig. 1.

The level of infection has usually been low; most fields have shown only a few bunted heads but an occasional field has been found with about 25% infection. In general, no appreciable losses in yield have occurred as a result of dwarf bunt, but its presence in the crop is nevertheless of economic importance, particularly to growers of winter wheat for seed. Registration or certification of seed is refused if dwarf bunt is detected in the field and confirmed by examining spores from the bunted heads.

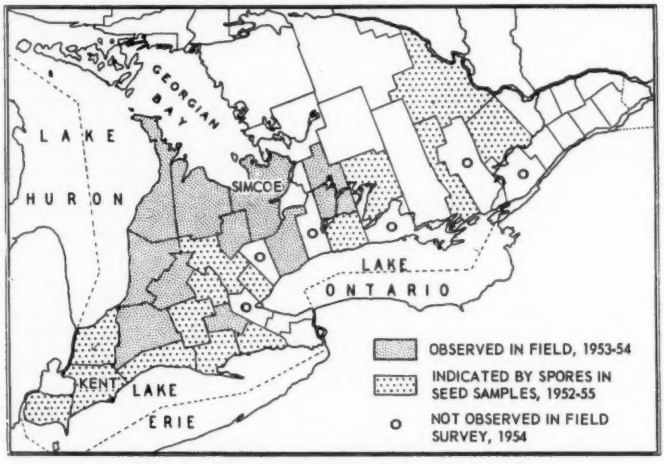


FIG. 1. Distribution of dwarf bunt in Ontario

Because of the scattered distribution and dwarf habit of infected plants in a field, the disease undoubtedly often escapes notice. During the years 1953 to 1955, all winter wheat seed which was submitted for registration was examined by the Plant Products Division for the presence of dwarf bunt spores. The results from some 2500 samples of seed examined indicated that dwarf bunt was present in 16 counties where the disease has not yet been observed in the field. In 1953, 12% of the samples were found infested with *T. contraversa* spores in trace amounts or more; and in 1954 and 1955, 10 and 9% respectively. Although only the better grades of winter wheat were thus sampled, 10% of the grain in the seed grades was contaminated in each of 3 consecutive years. A much higher figure might therefore be expected of the ordinary or commercial grades of winter wheat which are not subject to mandatory field or seed inspection for the presence of dwarf bunt. The commercial grade of wheat, however, is often used for seeding.

It has been assumed from general surveys that the disease is present in most areas of the province where winter wheat is grown, and furthermore that a considerable number of farmers each year grow wheat sufficient only for their own needs and without much regard for dwarf bunt contamination.

In order to determine exactly the incidence and severity of dwarf bunt in the province, a committee of growers and research workers of both the Canada and Ontario Departments of Agriculture decided to continue yearly observations by intensive surveys within specific districts. Two highly productive winter wheat areas were chosen: Simcoe County where the disease is well established, and Kent County where traces of dwarf bunt have been indicated only by seed examination.

Two detailed surveys in these areas have so far been made. The disease in 1955 was present more or less in trace amounts in 24% of all fields inspected in Simcoe County, whereas there was no indication of dwarf bunt in 178 fields of Kent County which were inspected by Dr. S. G. Fushtey of the Ontario Agricultural College. A similar level was obtained in 1956 when 20% of the fields surveyed in Simcoe were found infected but, as before, none were evident in the Kent County area. Spores of the dwarf bunt fungus, however, were detected in two seed samples taken from the 1956 harvest in Kent.

Experience in the United States has shown that dwarf bunt has become widespread and is the cause of heavy losses in areas of the Pacific northwest. In New York State the disease occurs in at least 10 counties adjacent to Lake Ontario. Tyler and Jensen, in unpublished data, stated that approximately 12% of the wheat acreage grown in that state for seed certification in 1952 was rejected because of dwarf bunt. European reports in general record great damage from dwarf bunt under various conditions. Losses in yield of between 50 and 70% have frequently occurred in recent years in the predominantly wheat-growing areas of Germany (20). Experience elsewhere has also shown that in a contaminated area a normal crop rotation cannot be expected to control dwarf bunt satisfactorily because the spores can survive over a long period in the soil. In Ontario then, where about 700,000 acres annually are planted to winter wheat, even the slightest occurrence of dwarf bunt must be regarded as a danger to the industry. The development of measures for the direct control of the disease is therefore an important consideration in an over-all program to combat dwarf bunt.

The Biology of the Dwarf Bunt Pathogen

Hosts

In Ontario *T. contraversa* has only been observed on winter wheat (*Triticum aestivum* L.). A few observations of its natural occurrence on native grasses in the United States have been reported (8), but as yet no grass hosts have been observed in Canada.

The species dates back at least to 1874 when Kühn in Saxony set up *T. contraversa* (14) for a smut collected on *Agropyron repens* (L.) Beauv. (*Triticum repens* L.). Connors (5) therefore suggested that the fungus may have been originally a parasite of this, or other native grasses in Europe, and later became pathogenic on wheat. Considerable evidence in support of this

theory has now been assembled by Duran and Fischer (6, 7), who have shown that *T. contraversa* is widely distributed on several grasses and cereals throughout the world.

Factors Influencing Spore Germination

Chlamydospores of dwarf bunt do not germinate under conditions ordinarily used for common bunt. Holton (11) succeeded in obtaining weak germination on water agar after 2 months' incubation at 10° C. with spores which had been presoaked for as long as 7 months in water. Gassner (9), Wagner (19), and others, however, were not able to duplicate Holton's results. Warmbrunn (21) also admitted that all attempts to germinate dwarf bunt spores had failed to produce any satisfactory results.

Lowther (15) was the first to demonstrate that spore germination of *T. contraversa* was dependent on low temperature. He was able to germinate untreated spores on water agar after 7 weeks at 5° C.; incubation at 10° C. resulted in no germination. In 1953, Gassner-Hoechst (9) germinated chlamydospores in soil-filled Petri dishes in an outdoor refrigerator exposed to daylight. He showed that germination commenced after 4 weeks under proper conditions of light, and temperatures close to 5° C. A maximum of 50 to 60% was reached in 10 weeks. Germination did not occur in total darkness. This method has since been corroborated by Böning *et al.* (2), Gassner and Niemann (10), and in the present work.

Materials and Methods

Fresh spore material for all experiments was obtained from dwarf-bunted heads of Cornell 595 which were kept at room temperature from the time of harvest each year.

In the preliminary experiments, the germination of chlamydospores was observed on 2% water agar directly under a compound microscope at weekly intervals during incubation at 5°, 10°, and 15° C. Germination included any stage of development of the promycelium arising from the chlamydospore (3). The controlled temperature chambers at first were in darkness, but later were fitted with artificial light. Incubation took from 8 to 12 weeks.

Subsequent spore germination tests were made on soil contained in standard Petri dishes of 20 mm. depth. A thin matrix of well-soaked peat moss was molded on the bottom half of the dish, over which was spread a uniform layer of dry sieved clay loam. The layer of wet moss was sufficient to moisten the soil to about 25% of its moisture-holding capacity, and the plates did not dry out during the long periods of incubation. More recently a layer of vermiculite has been used with better effect as a reservoir of moisture.

A spore suspension of one part by volume to four parts water was painted on the soil with a small camel-hair brush. The seeded plates were then exposed to light provided by fluorescent tubes in the temperature chambers. Where check series were to be kept in darkness, the plates were wrapped in tinfoil.

Spore germination, or sporidial formation, was observed weekly with a dissecting microscope under magnification 27× and was evaluated on a scale modified from that used by Gassner and Niemann (10) as follows:

Index of germination	Colorimetric description by Gassner and Niemann
0 = none	
1− = 1 to 10 spores per plate	
1 = approx. 20%	Weak gray, barely visible
2 = approx. 30%	Clearly gray
3 = approx. 40%	White-gray
4 = approx. 50%	Pure white, sporidia dense
4+ = over 50%	

Intermediate germination values were recorded by adding a plus (+) sign to the appropriate figure. The macroscopic appearance of maximum (4+) spore germination on soil plates is shown in Fig. 2.

Germination on Agar

During the preliminary tests on water agar it was discovered accidentally and quite independently of Gassner (9) that light greatly influenced the germination of the spores. It was necessary, during the tests which were set up in darkness at 5° C., to transfer some plates to a cool greenhouse at 7°–10° C. The plates which had been thus exposed to daylight from the 3rd week onwards all showed good spore germination in 5 weeks, whereas plates which had remained in darkness at 5°, 10°, or 15° C. showed no germination. The controlled temperature chambers were therefore fitted with lights and the experiments repeated. The results from three tests are shown in Table I.

TABLE I

PERCENTAGE GERMINATION OF *T. contraversa* CHLAMYDOSPORES ON WATER AGAR AFTER 8 WEEKS' INCUBATION AT DIFFERENT TEMPERATURES IN DARKNESS AND IN LIGHT

Temperature	Light source	Germination	
		Average	Maximum
Cool greenhouse 7–10° C. (Nov.–Dec.)	Daylight	37	58
	Dark	5	6
Constant temperature 5° C.	Artificial (40 ft-c.)	30	43
	Dark	5	7
Constant temperature 10° C.	Artificial	24	31
	Dark	5	7
Constant temperature 15° C.	Artificial	5	11
	Dark	Nil	Nil

By actual counts, approximately 50% germination was obtained on agar, which, when compared with Gassner's evaluation of germination on a soil substrate, was in all respects similar to his "pronounced" index of 4 obtained after 8 weeks at between 5° and 8° C. in daylight. No germination was

recorded for spores kept in darkness for the same period and temperatures. In general, the results of spore germination on agar indicated a definite response to daylight and artificial light, and were entirely comparable with the results that Gassner obtained on soil. All subsequent experiments were therefore set up on soil substrate.

Dependence on Low Temperature

It was convenient to use with some modifications the method on soil plates developed by Gassner (9). The minimum light intensity in the incubation chambers was also increased to between 150 and 200 ft-c. by use of fluorescent tubes.

TABLE II

AVERAGE GERMINATION OF CHLAMYDOSPORES ON SOIL PLATES AT CONSTANT TEMPERATURES IN CONTINUOUS LIGHT

Temperature, ° C.	Germination index—based on sporidial development				
	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks
0°	Nil	Nil	Nil	Nil	Nil
5°	2	3+	4	4	4
10°	1	1+	2	3	2+
15°	Nil	1−	1	1+	1−
Controls					
In dark at 5°	Nil	Nil	Nil	1−	1−

The germination optimum for chlamydospores on soil was determined within the limits of 0° and 15° C. as shown in Table II. The best germination occurred at 5° C. No germination occurred at 0°, and at 15° only a minimal number of spores had germinated in 8 weeks. While the interval between the time of painting the spores on the soil and the beginning of measurable germination at optimum temperature (5°) remained at 4 weeks, the peak value was often reached by the 6th week. An incubation period of 8 weeks seemed to be the limit for reliable determinations, and plates kept for periods of up to 12 weeks failed to show any increased germination. Contamination by moss or algae sometimes retarded the development of sporidia. Gassner has suggested that the growth of microflora like these might influence germination by the release of oxygen. In the present work, however, the microflora did not favor spore germination in soil plates. Furthermore the dissolution of sporidia, which occurs more rapidly at 10° C. or above, is a natural aging process whereby the fused primary sporidia disintegrate after sending out infection hyphae as they do on agar. Gassner and Niemann (10) in noting this phenomenon presumed that it was caused by the action of microorganisms in the soil.

Preconditioning Temperatures

Some stimulation of germination was noted as a result of freezing dwarf-bunted heads in ice for various periods before the start of tests, but a measurable effect could not always be obtained. Alternative methods of conditioning

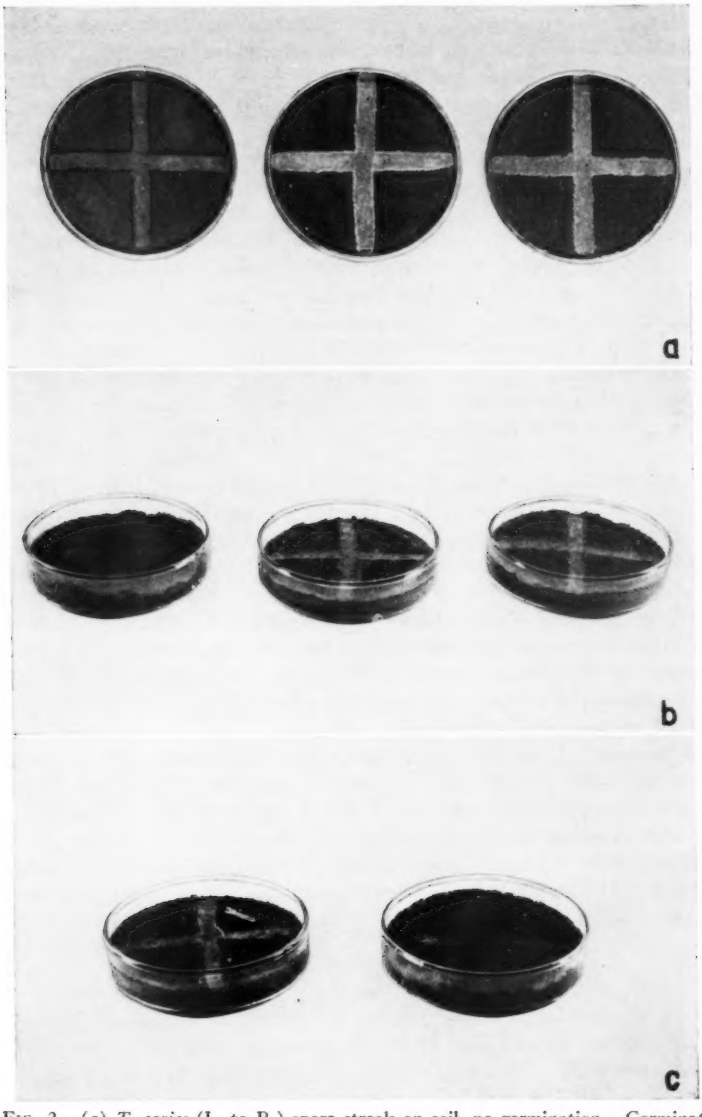


FIG. 2. (*a*) *T. caries* (L. to R.) spore streak on soil, no germination. Germination in other plates complete after 10 days at 10° C. in light and dark respectively. Germination index = 4+.

(*b*) *T. contraversa* spore streak on soil, no germination. Appearance of maximum germination after 8 weeks at 5° C. in light. Germination index = 4+.

(*c*) *T. contraversa* soil plates incubated 8 weeks at 5° C. in light and dark respectively. Germination index = 4+ and 0 respectively.

the spores to obtain maximum germination were therefore sought by varying temperatures as might occur in nature. In Fig. 3 are shown the significant differences in spore germination as a result of incubation on soil for 4 weeks in light at constant temperature, after which the plates were changed to another temperature at the time when the spores would normally start to germinate.

The only effect of exposure to a freezing temperature during the early part of incubation was that the start of germination was delayed by 1 or 2 weeks after the plates were placed at 5°, 10°, or 15° C. for the latter half of the incubation period. Exposure to above-optimum (10°) and maximum (15°) temperature for the first 4 weeks similarly retarded the beginning of germination. When the temperature was changed to 5°, however, germination was initiated and soon reached the equivalent of the controls held throughout at optimum temperature. In the plates which had been set up at 5°, germination had started at the time of changing to 10° and 15°. The germination index therefore was reduced in this case because of the slower development of sporidia at a higher temperature, and by the dissolution of the sporidia soon after they were formed.

It was concluded that maximum spore germination could only be obtained by exposure to a prevailing temperature of 5°, and was not improved beyond the established level of 50% by exposure to high or low temperatures during the first stages of incubation. The start of dwarf bunt in the field may thus be dependent on a mean temperature below 10° C. and any extreme deviation such as frost, or temperatures above 15° C., would merely delay the initiation. However, if germination has started, a prolonged exposure to temperatures above 10° C. would materially reduce the infective capacity of the fungus.

The influence of temperature variations upon spore germination was further tested by exposing the spores to differences of temperature between 0° and 15° C. which were alternated weekly for 5 to 6 weeks before the soil plates were finally held at constant temperature for the remainder of the incubation period. On the basis of weekly changes in temperature, the tests did not provide the desirable frequency such as must occur daily under natural conditions. The results, however, again emphasized the dependence of maximum germination on a prevailing temperature of about 5° C., and that periods of freezing do not materially reduce the capacity of the fungus to germinate at this temperature.

Dependence on Light

In 1954 Gassner and Niemann (10) showed that light had the greatest effect on germination when spores of the dwarf bunt fungus were exposed to light during the 5th and 6th week of incubation at 5° C. Their experiments made under continuous, but relatively weak, light of 90 to 140 ft-c. indicated a germination maximum of about 30%.

Throughout the present work, the Ontario collections of dwarf bunt have consistently shown a greater germinative energy than is recorded for the German material treated similarly by Gassner (9) and Gassner and Niemann

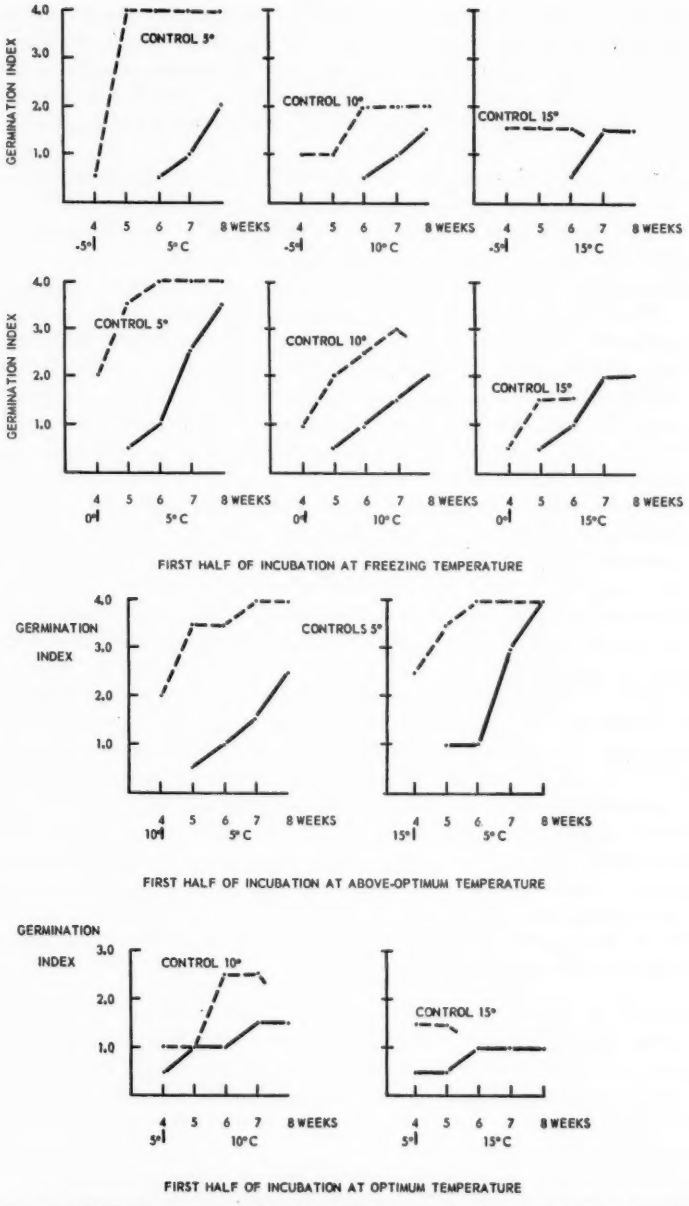


FIG. 3. Influence of preconditioning temperatures during first half of incubation period. Chlamydospore germination on soil plates in continuous light.

(10). On the basis of his own germination tests of Canadian material in 1954, the late Dr. G. G. Gassner-Hoechst conceded that the more rapid germination of the Canadian spores was of significance, and physiologically different from his own Bavarian collections.

In setting out to demonstrate the influence of light on the germination of the dwarf bunt pathogen, here it was soon apparent that a continuous exposure to fluorescent light of 150 to 200 ft-c. resulted in a much greater percentage of the spores germinating at 5° C. Ratings of over 50% (4+) were consistently obtained with chlamydospores which had been stored several months at room temperature. In general, the tests confirm the recent work by Gassner and Niemann, and therefore are reported only briefly.

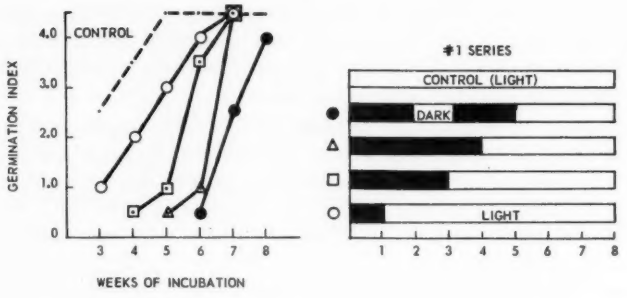
Soil plates were set up at 5° C. in three series to receive different exposures to the same light source. One series was held in complete darkness from the time the spores were painted on the soil, then at weekly intervals a number of the plates were exposed to light for the remainder of the incubation period. In the second series, the above procedure was reversed. The third series was set up with all plates in complete darkness for 3 weeks, then a third of the total number were exposed to light, each for 1, 2, and 3 weeks respectively, before being replaced in darkness. The differences recorded for germination in the three series are shown in Fig. 4.

Despite the fact that no visible signs are evident during the first 3 weeks of the germination process, the chlamydospores obviously become increasingly sensitive to light during this period. Increasing exposures to light thus resulted in an increased stimulation as measured by sporidial production. Maximum germination, as in the controls which were in continuous light, reached a peak at 5 weeks whereas only sporadic germination occurred in complete darkness. While the rapidity of sporidial development was particularly evident for spores which were first exposed to light during the 4th and 5th week of incubation, it is also obvious that some spores may germinate after a relatively brief exposure to light during the initial stages of incubation. In summary, however, the spores of *T. contraversa* on soil begin to germinate only after a conditioning period of 3 to 4 weeks at a favorable temperature.

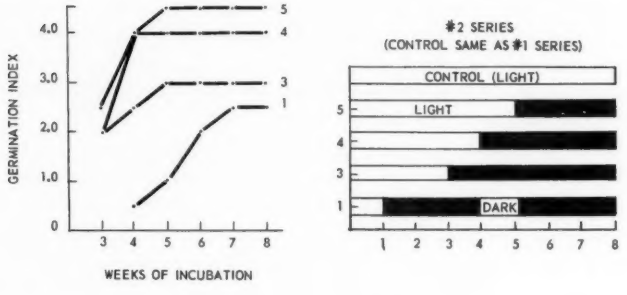
Factors Influencing the Incidence of Dwarf Bunt

The work of Young (22), Holton and Sunesan (13), and Rodenheiser and Holton (17) points to the fact that dwarf bunt infections arise mainly from the spores which are already in the soil, and not directly as a result of spores introduced with the seed. The disease is nevertheless spread to new areas in Ontario mainly by seed infested with dwarf bunt. Once the soil becomes infested, the ultimate appearance of the disease results, in a matter of time, by the interaction of host and pathogen as conditioned by environment.

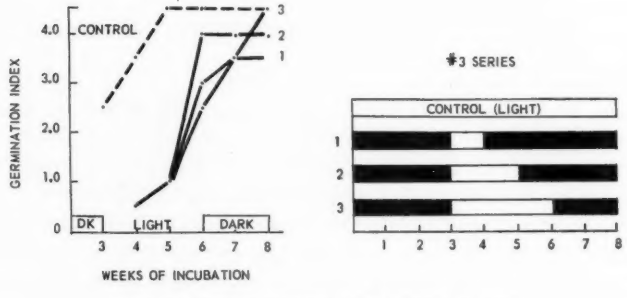
It has been observed in the United States and in Germany that in some years there may be a severe outbreak of dwarf bunt, while in others infection may be only slight (2, 12).



SET UP IN DARKNESS – THEN EXPOSED TO CONTINUOUS LIGHT



SET UP IN LIGHT – THEN PUT IN DARKNESS



SET UP 3 WEEKS IN DARKNESS – THEN EXPOSED TO LIGHT
FOR 1 TO 3 WEEKS ONLY

FIG. 4. Influence of light on chlamydospore germination on soil at 5° C.

To test the longevity of the Ontario dwarf bunt spores, dwarf-bunted heads of Cornell 595 were exposed to weathering in the field of their origin from October 1953. Whole heads and separate bunt balls from the 1953 harvest were enclosed in nylon cloth and placed on the soil surface, or were buried under 3 to 4 in. of soil to simulate as much as possible the conditions in infested soil. A sufficient number of heads were set out to provide for continuing yearly observations.

Germination tests of this material were made on soil plates at optimum temperature. With the exception of the separated bunt balls which disintegrated after 1 year and could not be recovered, the germinations in the 3rd year were of the same order as the 1-year tests. It is significant from the accompanying table that spore germination after 3 years' exposure to field conditions is relatively high and indicates that the chlamydospores may remain viable for a much longer period in the soil.

Dwarf-bunted heads collected 1953	Maximum germination in 8 weeks
Stored 3 years at room temperature	4+ (over 50%)
Weathered 3 years on soil surface	3 (approx. 30%)
Buried 3 years in soil, 4 in. depth	4 (approx. 50%)

Soil Type

The field surveys of winter wheat in Ontario since 1952 have associated the majority of dwarf bunt infections with heavy soils. Conners and Clark, who made the first survey in 1953, found the disease on almost every farm in an area of predominantly heavy soils except for two fields that were on light sandy soils. Holton *et al.* (12) have presented 15 years of field observations which suggest, however, that the disease may develop on both light and heavy soils in the Pacific northwest. Warmbrunn (21) also has observed that the disease occurs with equal intensity on sandy, organic, and light loam, as well as on the heaviest soils in the winter wheat areas of southern Germany. It would seem that the incidence of dwarf bunt is not greatly influenced by soil type. However, since most infections in the province have been observed on heavy soils, it must also be pointed out that winter wheat has largely replaced spring wheat in the province partly because it is adapted to the heavier soils which can more easily be worked in late summer or early fall.

At the beginning of the present investigations under controlled conditions, soil type was considered from the standpoint of getting successful spore germinations *in vitro*. The silt clay loam from an infested field in Bruce County proved superior to other soil types as a substrate because of its relative freedom from objectionable microorganisms. Garden compost was not as suitable and, since the heavy clay loam had already proved suitable for the development of dwarf bunt in the field, this particular soil type was therefore used throughout the spore germination tests.

Soil Acidity

Warmbrunn (21), after making extensive field surveys in Germany, stated that the heaviest infections of dwarf bunt were mostly on acid soils. The effect of soil acidity on chlamydospore germination was tested in the present studies by adjusting the normal soil reaction of the clay loam (pH 6.5) to acid and alkaline levels. Spores were incubated in light at 5° C. on soil adjusted to approximate levels of pH 5.0, 6.0, 7.0, and 8.0. No appreciable drift in the hydrogen-ion concentration occurred in the soil during the 8 weeks' incubation.

The results of two tests with spores of *T. contraversa* indicated that adequate germination (index = 3 to 4) occurred throughout the range of neutral to acid reaction, but at the alkaline level (pH 7.8 to 8.2) it was significantly reduced (index = 2). In additional tests with spores of *T. foetida* and *T. caries* at the same temperature, the level of germination was similarly reduced at about pH 7.8. Maximum germination of the common bunt spores, however, was complete within 14 days on soils between pH 5.0 and 7.0.

In the light of the reported observations of the natural occurrence of dwarf bunt on acid soils, it is significant that the fungus germinates effectively over a wide range of soil acidity.

Soil Moisture

Insofar as the writer has observed, dwarf bunt in Ontario is seldom related to a shaded or leeward situation which might influence the development of the disease by unequal snow cover. Tyler and Jensen (18), however, pointed to the frequently higher incidence of dwarf bunt on the leeward side of fence rows in New York.

The influence of soil moisture on the development of the disease along natural watercourses was sometimes strikingly apparent in fields surveyed in the province. From the number of infected plants which marked the edges of such channels it was presumed that the spores had been concentrated there by surface water. Significant too was the fact that although the dwarf-bunted plants appeared in different parts of a field with variable severity, the disease was never found in low areas or depressions which obviously were excessively moist.

In setting up the germination tests *in vitro*, it was necessary to give careful consideration to moisture content to prevent the soil from drying out during a long period of incubation. It was equally important for maximum germination that the soil should not be too wet. Based on many germination tests, an optimum moisture-holding capacity for the clay loam of about 25% was indicated. In excess of 40% moisture, the germination was reduced and readings were difficult because the sporidia seemed to disappear rapidly. The observations on soil moisture and its effect on spore germination *in vitro* are comparable with the observations on the influence of this factor on the development of the disease in nature.

The first field infection test made in 1953 determined, insofar as possible, the influence of soil moisture as well as temperature on the development of dwarf bunt. The results of this experiment in which dwarf bunt inoculum was applied to the soil were highly significant and have been published as a progress report (1). It is sufficient now to note the factors involved. Where spores were dusted on adequately moist soil immediately after seeding, a very high percentage of dwarf-bunted plants resulted. On the other hand, for adjacent plots similarly treated but on dry soil, there was very little infection produced. Under the conditions of the tests, it was assumed that the weather between seeding and the infection of the wheat is the chief factor in determining the severity of dwarf bunt. Böning *et al.* (2), however, assumed from their experiments that dry fall weather promoted dwarf bunt infection. On the basis of the present work, nevertheless, it was concluded that infection depended on the reserves of soil moisture being adequate to promote a sufficient and continuous germination of spores in the soil at a time when the wheat seedlings were susceptible.

Soil Temperature

On the assumption that the various soil conditions also act interdependently in the development of the disease, it is thus possible that infection might be reduced by the effect of a single factor in limiting fungus germination. Germination tests of spores *in vitro* showed that temperatures much above or below 5° C. retard the germination process. For maximum effect therefore, the initiation of germination in the field might reasonably be expected to occur at a mean temperature well below 15° and more likely below 10° C.

The results of the field tests planted in 1953 fortunately showed a correlation between temperature and the degree of bunt infection obtained. In the trials, two dates of seeding were used, September 12 and October 1. Mean daily air temperatures and rainfall were obtained from the official meteorological station records at Walkerton and Southampton in Bruce County. The air temperature recorded at the surface of the soil for 6 weeks after seeding compared well with the official records taken within 20 miles of the tests.

The mean maximum temperature for September was 21° C. and the mean minimum 10.5° C. However for the 4-week period beginning September 12, the daily mean temperature was 15.5° C., which corresponded to the maximum limiting temperature for spore germination *in vitro*. Corresponding mean temperatures for October were 17° and 4.7° C., and a daily mean of 8.6° C. for the 4 weeks from October 1. Obviously the mean daily temperature in October was favorable for the initiation of germination, a factor which, combined with the effects of soil moisture, undoubtedly contributed to the extraordinary severity of the resulting dwarf bunt infections in the plots that were sown in that month.

Light

As already stated in the design of the field experiments, the spores of *T. contraversa* were dusted over the surface of the plots immediately after seeding.

Since the germination process is influenced by light, it was expected that the largest amount of infection would result from this treatment in comparison with others. Significant increases of up to 30% infection were in fact realized. In contrast, inoculum that was mixed with the seed before sowing, or spread in the open drill, did not significantly increase the amount of infection nor was it expected to, since controlled experiments had proved that the dwarf bunt spores do not normally germinate in continuous darkness.

Summary and Discussion

To study some of the irregularities noted in the occurrence of dwarf bunt it was imperative to examine the influence of environment on host and pathogen. To begin with, the chlamydospores of *T. contraversa* do not germinate readily and require a conditioning period of 3 weeks or more incubation at relatively low temperature before even starting to form sporidia. A temperature of 5° C. throughout 8 weeks' incubation proved optimum for best germination, whereas sustained temperatures much above or below 5° C. not only slowed development of the sporidia, but at higher temperatures contributed to their rapid exhaustion, thereby reducing the infective capacity of the pathogen.

The entire germination process is further influenced by a dependence on light for, in the absence of daylight or artificial light, only occasional chlamydospores are able to germinate regardless of a favorable temperature. On soil plates, the spores become sensitive to light during the initial stage of incubation starting at about 3 weeks. Exposure to light from then onward results in the most stimulus to germination. A brief exposure to light only during the latter half of incubation is therefore an effective stimulus for the development of sporidia. Chlamydospores at optimum temperature, thus sensitized for as little as 1 week in light, are subsequently able to germinate in darkness.

Because of a dependence on light for germination, infection of wheat is most likely to occur at or near the soil surface from spores that have been conditioned on the soil and which can survive at least 3 years in soil without much loss of viability. Incidental factors favorable to spore germination are a somewhat acid soil reaction and an adequate, but not excessive, amount of soil moisture. The observations made *in vitro* are completely substantiated by field experiments which showed the interdependence of these individual factors in their influence upon spore germination and the infection resulting therefrom.

A correlation was shown to exist between temperature, light, and soil moisture, and the severity of dwarf bunt infection in field plots. Two seeding dates were used but the climatic conditions which prevailed from the time of seeding until infection proved more important than the actual seeding date. Wheat sown in September on heavily infested soil was not severely infected because spore germination was almost inhibited by warm

temperatures and an inadequate reserve of soil moisture. The same conditions, however, permitted quick emergence and development of the seedlings beyond a stage when they were susceptible to infection. In contrast, the cool temperatures and moist soil condition which attended the seeding in October were such that most seedlings became infected, and the majority of plants were severely diseased. It was concluded that in this case the susceptible stage of the host was during the period of seedling emergence which must have coincided with a maximum spore germination beginning about October 22.

The extraordinary severity of dwarf bunt which was thus brought about was indicative of a rather exceptional combination of climatic factors which favored germination and infection by the pathogen. It is obvious, too, that these same factors of light, temperature, and moisture so necessary for the effective germination of the dwarf bunt spores will vary considerably from one season to another, or even at different places in the same season. Seasonal variation might therefore account for the irregular incidence of the disease as reported in the literature. It might also be partly responsible for the different observations which point to severe dwarf bunt infections resulting from early seeding of fall wheat (2, 10).

It must be conceded that it may not be essential for dwarf bunt spores to germinate only at the soil surface. In the normal preparation for seeding, spores which are turned under after a conditioning period on the soil might germinate well enough below ground under the lasting stimulus of light. Indeed there is ample evidence to support this alternative in the severe infections which resulted only from the natural soil infestation at the time of the field tests (1).

Insofar as most efforts are being concentrated on the development of wheats resistant to common bunt as well as the dwarf bunt fungus, the present application of these observations lies in the development of a method of testing winter wheat for dwarf bunt resistance under controlled environment.

Acknowledgments

The writer is indebted to Mr. I. L. Conners for his original collections of dwarf bunt material and for many helpful suggestions, also to Drs. F. L. Drayton and R. A. Shoemaker for helpful criticism and discussion of the manuscript.

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REGENERATION IN SPLACHNUM AMPULLACEUM (L.) HEDW.[1]

KRAFT E. VON MALTZAHN AND MARY M. MACNUTT

Abstract

Successive leaves isolated from the gametophore exhibit differences in the number of regenerates obtained per organ. Starting from the base, successive leaves show an increasing number of regenerates per leaf. This pattern is similar in leaves isolated from young and older gametophores, although the younger ones show a much greater absolute number of regenerates.

No correlation exists between the number of cells per organ and the number of regenerates. Although the size of the wound surface plays an important part in the number of regenerates produced, this factor cannot determine the regenerative pattern between successive leaves.

Correlative factors are shown to exist in individual leaves and the distribution of regeneration in the leaf is determined also by these factors.

It is concluded that the age of the organ determines the regular pattern of protonematal regeneration of successive organs.

Introduction

Mosses exhibit generally great potentialities for regeneration. Experimentally these can be realized by means of isolating parts of the whole. This indicates that correlative factors between parts of the whole gametophore inhibit regeneration and that a disturbance of natural correlations, e.g. by means of the isolation of parts, will remove this inhibition. The regenerative behavior of different parts of the gametophore may therefore be an indication of the distribution of correlative factors in the plant.

Not every cell, however, shows the same potentiality of regeneration after isolation. The regenerative behavior will not only depend on correlative factors, but also on the developmental state of the part. The present study is concerned with the problem of the distribution of regenerative potentialities and their respective realization in the moss gametophore.

Materials and Methods

Throughout the present study the male strain of *Splachnum ampullaceum* was used. The plant material has its origin from the Cambridge (England) Collection. Leaves were isolated from the gametophore; those directly at the apex were too small for dissection and were not included in the present observations. The isolated leaves were grown under sterile conditions on Beijerinck's inorganic medium with 0.1% sucrose and 1% agar at pH 6 and under daylight fluorescent lamps. Each datum represents the average obtained from 10 to 12 plants.

Results

Comparing the behavior of successive leaves isolated along the axis of the gametophore, it was found in each case that the relative number of regenerates between successive leaves did not change with time, although the absolute number per leaf increased with time. Regeneration of successive leaves

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Contribution from the Botanical Laboratories, Dalhousie University, Halifax, Nova Scotia. This study was carried out with the assistance of a grant from the National Research Council of Canada.

may therefore be compared after the same absolute time interval. Leaves were isolated and the number of regenerates was generally counted 4 days after planting.

The results of a typical experiment are described in Fig. 1. Starting from the base, successive leaves show an increasing number of regenerates per leaf.

In a number of similar experiments, however, considerable variation in the absolute number of regenerates was found to occur. Since the age of the gametophores was not considered in the above experiments, it seemed to be necessary to test whether such a variation may be due to differences in the ages of the gametophores.

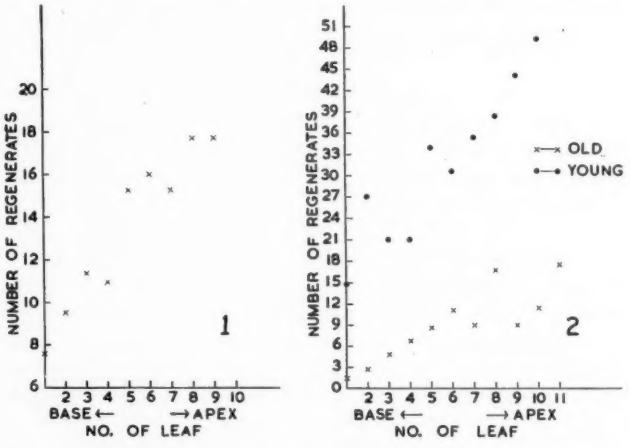

FIG. 1. Protonematal regeneration from successive leaves.
FIG. 2. Protonematal regeneration from successive leaves from young and older gametophores.

Successive leaves were isolated from young and older gametophores and their regenerative behavior compared. Fig. 2 gives the results of this experiment. In each case there is an increase in the number of protonematal regenerates per leaf from the base to the tip of the gametophore. The absolute number of regenerates of the same leaf is rather different, though, depending on the age of the gametophore. Leaves from younger gametophores show consistently a much higher number of regenerates compared to leaves from older plants.

It may be suggested that differences in the number of regenerates in successive leaves are due to differences in the number of cells per leaf from which regeneration may occur. To test this factor leaf size and cell size of successive leaves were determined. Cell size of comparable regions was approximately the same between successive leaves. Leaf size, expressed in terms of leaf length and width, showed considerable differences. This is represented in Figs. 3 and 4. Particularly, successive leaves at the base show differences

in mature size. Since cell size was approximately the same, differences in the size of successively formed mature leaves must be due to the number of cells per leaf.

Since the curve for the number of protonematal regenerates per successive leaves does not show any similarity to the curve for the size of successive leaves, the size of successive leaves cannot account for the pattern of protonematal regeneration from successive leaves.

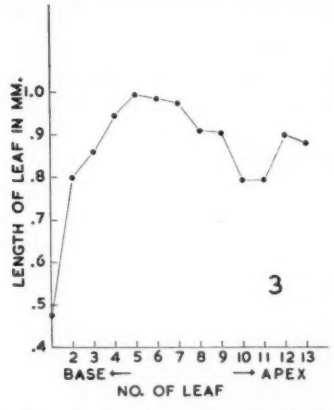

FIG. 3. Length of successive leaves.

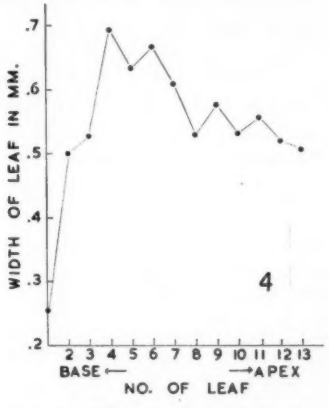

FIG. 4. Width of successive leaves

It is also important to investigate the influence of the size of the wound surface on regeneration. For this question the regenerative behavior of whole isolated leaves was compared with that of leaves cut into halves along the longitudinal axis and those in which two cuts were performed at right angles to the longitudinal axis dividing the leaf into three parts. The following values were obtained:

	Number of protonematal regenerates per leaf
Whole leaf	31.5
Leaf cut into two halves (along longitudinal axis)	42.6
Leaf cut into three equal parts (proximal, middle, distal)	49.7

Evidently the size of the wound surface plays an important part in determining the number of regenerates per leaf. Since the wound surfaces between successive leaves which have been isolated are of approximately the same size, differences in the regenerative behavior of successive leaves cannot be due to differences in the size of the wound surface.

Not only correlative influences between successive leaves have to be considered but also correlative influences between different parts of the same leaf. Whole leaves were marked into three zones (I distal, II middle, and III proximal). The regenerative behavior of these zones in whole leaves is compared with that in which these zones are isolated. Figs. 5 and 6 give the results of these experiments. Zone II shows the greatest amount of regeneration, while both Zones I and III lag behind Zone II. This would be an indication that these leaves do not show polarity in terms of their regenerative behavior.

When the zones are isolated the zonal behavior changes. Pieces corresponding to Zones I and III pick up and show approximately the same number of regenerates as does Zone II. Zone II apparently inhibits the realization of regenerative potentialities of Zones I and III to some extent. Once the zones are isolated, this inhibition is removed and activation takes place.

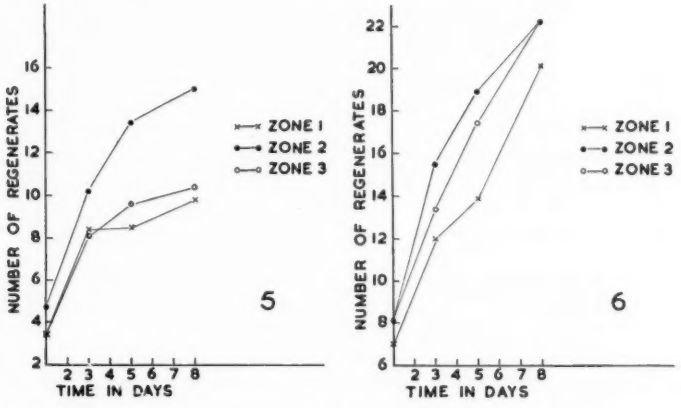

FIG. 5. Protonematal regeneration from three zones of whole leaves.
FIG. 6. Protonematal regeneration from three isolated zones of leaf corresponding to the zones of Fig. 5.

Discussion

Correlative factors are responsible for the inhibition of regeneration in whole intact plants and for the distribution of regeneration in whole detached leaves. The distribution of regeneration in successive leaves, on the other hand, does not seem to be a function of the position of the leaf in the whole gametophore or mature size of the leaf, but rather of the developmental stage or age of the organ. This conclusion is further supported by the fact that differences in the age of the whole gametophore will have a considerable influence on the regenerative behavior of comparable isolated parts.

Working with isolated leaves of the gametophore of *Funaria hygrometrica*, Bopp (1) did not find a progressive decrease of regenerative powers of the leaves from the tip to the base of the gametophore. The leaves located toward the middle of the stem gave the smallest number of regenerates.

In his material, therefore, primarily position of the leaf in the gametophore, not age, determined the number of regenerates per leaf. Gemmell (2) found also a second maximum in regeneration, in similar experiments with detached leaves of *Atrichum undulatum*, so that in this material position also and not only age seems to be important. Leaves of *Splachnum ampullaceum* seem to show strictly age dependence in their regenerative behavior in contrast to both *Funaria* and *Atrichium*.

Porzer (5) studied the stainability of leaves of foliose Hepaticae with alkaline dyes. He found a gradient in stainability from very young to the older leaves, indicating a change in terms of certain cellular conditions with an increase in the age of the organ. Similar results were obtained by Kressin (3) (see Strugger (6)).

The development of regenerative protonemata in the present material was not confined to one particular portion of the leaf, although the middle region showed a higher number of regenerates. This was found to be due to correlative factors, since after isolation of the distal, middle, and proximal regions, the distal and proximal ones caught up with the middle zone in the number of regenerates, suggesting the presence of an inhibitory influence on distal and proximal parts by the middle zone, which is removed by means of isolation.

Meyer (4) studied the regenerative behavior of leaves of *Physcomitrium turbinatum*. Protonematal regeneration was not confined to any particular part of the leaf. Bopp (1) showed the distal end to be the predominant location of regeneration in detached leaves of *Funaria*, the proximal region in those of *Tortula muralis*. In *Funaria*, isolation of the regions led to an increase in regeneration. He explained this observation also by means of a removal of inhibitory influences brought about by isolation. Different mosses apparently show quite a different behavior.

Predominant region of leaf in which regeneration takes place:	
Distal	*Funaria hygrometrica* (after Bopp)
	Atrichum undulatum (after Gemmell)
Middle	*Splachnum ampullaceum*
Proximal	*Tortula muralis* (after Bopp)
Region not defined	*Physcomitrium turbinatum* (after Meyer)

In each case in which a predominant region for regeneration is present, isolation of the regions leads to an increase in the number of protonematal regenerates in the inhibited zones due to a removal of the inhibitory influences.

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THE GENETICS OF SELF-INCOMPATIBILITY IN LESQUERELLA DENSIPILA AND IN THE F_1 HYBRID L. DENSIPILA × L. LESCURII[1]

D. R. SAMPSON[2]

Abstract

The genetics of self- and cross-incompatibility was studied in 12 related families of *Lesquerella densipila* and in three families of the F_1 hybrid *L. densipila* × *L. lescurii*. Silique swelling was used as the criterion for compatibility. Incompatibility results from the failure of pollen tubes to penetrate the stigmatic surface. Incompatibility in *Lesquerella* is controlled by a series of multiple *S*-alleles at one locus. The pollen phenotype is determined sporophytically. A minimum of eight *S*-alleles was obtained from *L. densipila*, and two from *L. lescurii*. Two sorts of gene interaction, independence and dominance, were found among the fully analyzed heterozygotes. Dominance was restricted to the pollen controlling part of the alleles and two levels of potency were established.

Introduction

Self- and cross-incompatibility systems in higher plants can be divided into two broad categories depending on whether the incompatibility phenotype of the pollen is determined by the genotype of the pollen grain itself (gametophytic systems) or by the genotype of the pollen producing plant (sporophytic systems). For many years it was thought that all incompatibility systems which were controlled by multiple alleles belonged to the gametophytic type, and that sporophytic systems were restricted to heterostylic species. The combination of multiple alleles with sporophytic determination of the pollen behavior was first demonstrated in 1950 (3). In 1954, Bateman (1) established that *Iberis amara*, a member of the Cruciferae, had a sporophytic system with multiple alleles. Further work, and the reinterpretation of old data, showed that this also applied to *Brassica campestris*, *Cardamine pratensis*, *Capsella grandiflora* (2), *Brassica oleracea* (7, 8), and *Raphanus sativus* (6). Thus, the work on *Lesquerella* brings to six the number of cruciferous genera in which a multiple allelic, sporophytic system of incompatibility has been found. Since *Lesquerella* belongs to a different tribe (Drabeae) than the other five, the idea that self-incompatibility throughout the Cruciferae is due to the same mechanism (2) is considerably strengthened.

Materials and Methods

This study began with six plants which were grown from seed collected by Rollins (5) in the Nashville Basin of Tennessee. Parents 1, 2, 3, 4, and 5 belong to *L. densipila* Rollins (population 131). Both species have $2n = 16$ chromosomes (5). All plants were grown in pots in an insect-free greenhouse which was kept at temperatures above 65° F. Experiments were conducted under natural day lengths from January to June at Cambridge, Mass.

[1]Manuscript received May 10, 1957.
Contribution from the Biological Laboratories, Harvard University, Cambridge, Mass.
[2]Formerly Research Assistant, Bussey Institution of Harvard University. Present address, Horticultural Division, Central Experimental Farm, Ottawa, Ont.

The study was primarily concerned with demonstrating the type of incompatibility system in the material, and with determining the genotypes of the mating groups and the activity (phenotypes) of the incompatibility alleles. Compatibility relationships of the parent plants were determined in 1954. This showed only that self- and cross-incompatibility existed in the material. Most of the data on the genetics of the system were collected from progeny families raised the following year. Unfortunately, this material was originally designed to detect a novel type of gametophytic system which was described in *Brassica* by Kakizaki (4) but never found since, not even in *Brassica*. Bateman's paper (1) came to hand too late to prepare thoroughly for a sporophytic system. Accordingly, after the analysis of a few progeny families had indicated a sporophytic system in *Lesquerella*, genotypes were assigned to the mating groups of characteristic families. Genotypes of certain parents could then be deduced from recombinations occurring in the progeny. Proof of this interpretation utilized the method of prediction, for, with the genotype of a parent established from the data of one family, characteristics of related families could be predicted and experiments carried out to test the predictions. The analysis of these families, in turn, provided the basis for further predictions. The argument constructed was reticulate rather than linear with numerous cross-checks that added confidence to the interpretation.

The only experimental data available was whether a pollination was compatible (+), incompatible (−), or intermediate (±). All else was deduced from genetic relationships. Unless otherwise noted, all pollinations were made with freshly dehisced pollen and 1- or 2-day-old stigmas. The compatibilities of the parent plants were determined by the amount of ripe seed produced (Table I). However, effective analysis of compatibility relationships in progeny families demands a rapid method for determining pollination results so that critical combinations can be achieved before the plants die. In the *Lesquerella* species and hybrids studied, siliques enlarge rapidly following compatible, but remain small after incompatible, pollinations. Therefore, silique swelling was used as the criterion of compatibility, and readings were taken 4 days after pollination. Five flowers were used for each pollination. If four or more siliques developed, the cross was considered compatible; if four or more failed, the cross was incompatible. When the results were indecisive, the pollination was repeated. As all plants were self-incompatible, emasculation was not practiced. Forceps were sterilized in absolute alcohol after each pollination.

Self- and cross-incompatibility in the *Lesquerella* material, as will be shown later, results from growth inhibition of incompatible pollen tubes on the stigma surface. Therefore, both the production of ripe seed and silique swelling give indirect evidence on the phenomenon under study. Possibly silique swelling, because it is nearer in time to pollination, is the more reliable of the two methods (cf. stigma flushing (1)). For example, the development of parthenocarp siliques in interspecific crosses involving *L. grandiflora* was reported by Rollins (5). This was confirmed for the cross *L. grandiflora*×*L. densipila*.

However, close examination of enlarged siliques showed the presence of tiny aborted seed, indicating that fertilization occurred. Had ripe seed data been used, the failure of this cross might have been attributed to pollen–stigma incompatibility rather than to somatoplastic sterility.

Several factors influenced silique swelling. Vegetative weakness produced by (*a*) old age, (*b*) severe infection of powdery mildew, (*c*) extreme inbreeding depression, and (*d*) lack of sunshine on 17 successive rainy days resulted in failure of siliques to develop following compatible pollinations. Also, the presence of a large number of developing siliques on a plant reduced the success of further compatible pollinations. Accordingly, no more than 20 flowers per plant were pollinated at one time. When the results of compatible pollinations were noted, the developing siliques were removed. No condition was discovered which favored silique development following incompatible pollination of anthesis.

Most of the data from progeny families are given in checkerboard diagrams. Females are entered at the top of a diagram and males at the side. The second column and row of a diagram contain the individual plant numbers. The first two figures of a progeny plant number indicate parentage. For example, the first plant of family 2×4 is numbered 241, the second, 242. The prefixed family numbers are frequently omitted from a diagram; then the family is indicated by the upper left box heading. None of the parents was tested against a progeny plant nor entered in a diagram.

The first column and row of a diagram contain the allele subscripts representing the *S*-genotype of an individual or class. A question mark signifies an unidentified allele. Phenotypes are indicated by addition of symbols to allele subscripts. An active allele is denoted by a dot superscript, a recessive allele by parenthesis, and an allele of unknown activity is unqualified.

Results and Interpretation

Parent Plants

The five original plants of *L. densipila* were selfed and crossed to each other in 14 combinations. All five plants were self-incompatible, parents 1 and 3 were mutually cross-incompatible in both directions, and the remaining pollinations were compatible (Table I). In all, four seed-bearing siliques developed from 98 flowers pollinated with incompatible pollen. This indicates a low percentage of pseudocompatibility. On the other hand, 82 siliques developed from 98 flowers pollinated with compatible pollen.

The one plant of *L. lescurii* was not hand-selfed, but set no seed on isolation. It was crossed to some of the five plants of *L. densipila* in seven combinations, all of which were compatible. From 62 flowers pollinated, 56 siliques developed.

Stigmas of both species, squashed in a drop of acetocarmine, were examined microscopically 24 hours after pollination. It was observed that incompatible pollen either failed to germinate or produced very short tubes which did not

penetrate the stigmatic surface. Compatible pollen germinated and penetrated readily. Similar observations on the immediate cause of incompatibility have been made on all other crucifers so examined.

Four plants were successfully selfed by bud pollination (Table I). Freshly dehisced, incompatible pollen was applied to the stigmas of 118 unopened flowers and 69 siliques developed. By tagging bud-selfed flowers and noting when they opened, it was observed that the best results were obtained by pollinating 2 or 3 days before the buds opened. The incompatibility mechanism appears to be fully developed from 48 to 36 hours before the buds open. Similar results are well known in *Brassica*.

The progeny families will now be considered. Twelve of these were derived from the five parent plants of *L. densipila* and three were from the one plant of *L. lescurii* crossed with three of the *L. densipila* parents.

TABLE I

RESULTS FROM SELFING AND CROSSING *Lesquerella* PARENT PLANTS

Pollination	No. flowers pollinated	No. siliques developed	Av. no. seeds per developed silique
1×1	22	0	0.00
1×1 bud	14	10	3.75
1×2	7	6	3.33
1×3	11	0	0.00
1×4	2	2	3.50
2×2	12	0	0.00
2×1	9	9	4.70
2×3	3	3	4.00
2×4	6	6	5.67
2×5	7	7	5.40
2×6	7	7	5.28
3×3	18	0	0.00
3×3 bud	10	7	2.00
3×1	22	2	1.00
3×2	13	11	3.70
3×4	5	4	4.00
3×5	6	6	5.00
3×6	4	3	7.50
4×4	6	1	2.00
4×1	10	5	1.66
4×2	25	19	5.52
4×5	5	4	2.00
4×6	7	6	3.50
5×5	7	1	0.00
5×5 bud	52	29	4.59
5×6	2	2	—
6×6 bud	42	23	2.10
6×1	3	3	1.00
6×3	37	33	2.80
6×5	2	2	2.50

Families 2×4 and 4×2

The progeny analysis began with family 2×4 which contained 44 plants. Seventeen plants were tested for self-compatibility but no siliques developed; the remaining plants developed no siliques spontaneously upon isolation.

Intersib pollinations in family 2×4 showed that plants 241 and 245 were mutually compatible in both directions. However, both these plants were self-incompatible and, since the pollen of each behaved differently on 241 than on 245, the two plants represented two mating groups. Their pollen was used to divide the entire family into two classes of 26 and 18 plants each (Fig. 1). In an attempt to reveal further classes, 17 sibs were crossed with each other in 246 combinations (Fig. 3). The attempt failed. The two classes were completely intra-incompatible, and inter-compatible in both directions.

Family 4×2 was also divided into two classes by the pollen of 241 and 245 (Fig. 4). Nine sib combinations were made. These showed that the compatibility behavior of the two classes of 4×2 was the same as the behavior of the 2×4 classes.

When a particular family and its reciprocal contain the same two mating groups, the data can be explained on the basis of a gametophytic incompatibility system only if the parents had no common incompatibility allele and one parent had been homozygous. Thus:

$$S_1S_1 \times S_2S_3 \rightarrow S_1S_2,\ S_1S_3$$

$$S_2S_3 \times S_1S_1 \rightarrow S_1S_2,\ S_1S_3$$

The two groups resulting from each of these crosses would behave exactly like the classes of 2×4 and 4×2. However, as the homozygous parent could arise only by pseudocompatibility, it appeared improbable, but still possible, that a gametophytic system was operating in *Lesquerella*.

Family 3×5

As families 2×4 and 4×2 yielded limited information on the type of incompatibility under study, a fresh start was made on the 20 plants of family 3×5. The pollen of 351 divided the family into two classes and the pollen of 354 confirmed the division (Fig. 2). When the family was tested by 351 as female, a third class was revealed. The pollen of 353, a member of the third class, completed the division into four classes. A tester from each class was used on the remaining plants of the family in both directions with the results from all but one of the 144 combinations agreeing (Fig. 2).

A family with a gametophytic type of incompatibility would contain four classes if both parents had been heterozygous and had no alleles in common. However, all interclass combinations of such a family would be compatible. Since family 3×5 did not fit this pattern, a sporophytic system was suggested and genotypes were assigned to the four classes on this basis.

The assignment of genotypes utilized the "compatible diagonal" method (1). First it was assumed that incompatibility resulted when the pollen and

2 × 4	1	2	3	4	7	8	9	10	12	13	14	15	16	19	20	21	23	25	30	35	36	39
(♀)241	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
(♂)245	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

2 × 4	40	41	43	44	5	6	11	16	17	22	24	26	27	28	29	31	32	33	34	37	38	42
(♀)241	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(♂)245	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

1

3 × 5		14	15	48	58	14			15					48				58		
		353	356	351	354	14	16	19	7	8	11	17	18	2	5	9	13	15	10	12	20
1(4)	353	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
1(5)	356	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
48	351	−	+	−	+	−	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+
58	354	+	−	+	−	+	+	+	−	−	−	−	±	+	+	+	+	+	−	−	−
1(4)	14	−	−	+	+																
	16	−	−	+	+																
	19	−	−	+	+																
1(5)	7	−	−	+	+																
	8	−	−	+	+																
	11	−	−	+	+																
	17	−	−	+	+																
	18	−	−	+	+																
48	2	−	+	−	+																
	5	−	+	−	+																
	9	−	+	−	+																
	13	−	+	−	+																
	15	−	+	−	+																
58	10	+	−	+	−																
	12	+	−	+	−																
	20	+	−	+	−																

2

2 × 4		2♀												3♀				
		1	2	3	4	7	8	9	10	12	13	14	15	5	6	11	16	17
2♀	1	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	2	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	3	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	4	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	7	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	8	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	9	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
	10	−	−	−					−				−	+	+	+	+	+
	12	−	−	−						−			−	+	+	+	+	+
	13	−	−	−							−		−	+	+	+	+	+
	14	−	−	−								−	−	+	+	+	+	+
	15	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
3♀	5	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
	6	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
	11	+	+	+									+	−	−	−	−	−
	16	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
	17	+	+	+	+								+	−	−	−	−	−

3

4 × 2	1	2	4	7	11	12	13	15	17	18	21	22	23	24	26	3	5	6	8	9	10	14	16	19	20	25
(♀)241	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
(♂)245	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−

4

FIG. 1. Classification of family 2×4 into two groups.
FIG. 2. Sib combinations in family 3×5.
FIG. 3. Sib combinations in family 2×4.
FIG. 4. Classification of family 4×2 into two groups.

stigma concerned had at least one active *S*-allele in common. Secondly, it was assumed that parents 3 and 5 were both heterozygous for *S*-alleles, and yet had no alleles in common. Thus the four classes in family 3×5 should represent the Mendelian recombination of the putative four parental alleles. If the premises and conclusion are correct, it follows that each progeny class will share no alleles with only one other class, and thus it will be compatible with that class in both directions. The results from the 16 crossings and selfings possible with four classes were then arranged on a checkerboard so that the diagonal from top right to bottom left contained only compatible crosses (Fig. 16). When such a "compatible diagonal" is achieved, and the order of the classes in the column is the same as in the row, and the data are such that no two classes at either end can interchange positions without disrupting the diagonal, then the four compatible pollinations on the diagonal represent the crosses between classes with no common alleles. Thus it was deduced from the Mendelian laws and the arrangement of the classes of 3×5 on the checkerboard that the classes of 353 and 356 had one allele from one parent while those of 351 and 354 had the alternate allele of that parent. Similarly the classes of 353 and 351 had one allele of the second parent while those of 356 and 354 had the alternate allele of the second parent. One parent was assigned the genotype S_1S_8, and the other S_4S_5; the progeny classes of 353, 356, 351, and 354 were given the genotypes S_1S_4, S_1S_5, S_4S_8, and S_5S_8 respectively (Figs. 2, 16). The observed ratio of 4:6:6:4 was close enough to the expected 1:1:1:1 to provide confidence in the system. Furthermore the assigned genotypes were definite enough to be subjected to further test.

Family 5×5

The four plants derived from parent 5 by bud pollination provided the first test of the interpretation of family 3×5. Family 5×5 originally contained 120 plants. These were planted in June as part of an experiment to test the hypothesis of Kakizaki (4), but *L. densipila* was found to be a short-day plant and did not flower during the summer. Only four plants survived until they could be used the following February. The four were then very weak and proved useless as female testers because, with compatible pollinations, perhaps but one of the five flowers pollinated would develop into a silique. They were used as male testers, but not until it was shown that their pollen was viable and the incompatibility mechanism of the pollen was unimpaired.

Pollen of the four plants of family 5×5 was used on each of the four class testers of family 3×5 (Fig. 11). Three of the four were entirely incompatible. To determine whether this was due to sterility caused by vegetative weakness, the pollen of 551 was tested on 241 and that of 554 was tested on 241, 246, 321, 322, 327, and 112. As all seven combinations were fully compatible, the behavior of 551 and 554 toward the classes of family 3×5 must be attributed to incompatibility. The pollen of 552 was more revealing, being compatible on 353 and 351 but incompatible on 356 and 354.

The interpretation of family 3×5 assigned the genotypes S_1S_8 and S_4S_5 to the parents but did not state which parent had which genotype. However, since 552 was incompatible on 356 and 354, which had S_5 in common, it follows that 552 had S_5 and that parent 5 must have been S_4S_5. Further, parent 3 must have had the genotype S_1S_8, 554 that of S_4S_5, and 552 that of S_5S_5. Thus family 5×5 not only confirmed the assignment of genotypes to family 3×5 but also provided the information necessary to establish the genotypes of the parents. Its contribution to understanding allele activity will now be considered.

Allele Activity in Families 3×5 and 5×5

The genotypes of five mating groups of *L. densipila* are now established. Determination of phenotypes is more difficult because a given allele may act differently in the pollen than in the stigma, and it may be recessive to a second allele but independent of, or dominant over, a third. These differences cannot be predicted with confidence. Therefore, phenotypes can only be established by experiment.

When the genotypes of classes were worked out, and two classes having only one common *S*-allele were found to be mutually cross-incompatible, the common allele was assumed to be active in the pollen and stigma concerned. If, however, the classes were compatible in one direction, the allele may have been active in the stigma but recessive in the pollen, or active in the pollen but recessive in the stigma, or recessive in both. In such a situation, where the precise activity is unknown, the allele involved is said to be "somewhere" recessive. Whether it is recessive in the pollen or stigma, or both, can only be determined by testing each sex by the opposite sex known to have that allele active.

Intersib combinations in family 3×5 showed that S_1 was active in the pollen and stigmas of S_1S_4 and S_1S_5 because classes with these genotypes were mutually incompatible in both directions (Fig. 2). Also, S_4 and S_5 were active in the pollen of S_4S_8 and S_5S_8 and the stigmas of S_1S_4 and S_1S_5. However, four compatible combinations between genotypes with common alleles (Fig. 2) indicated that S_4, S_5, and S_8 were recessive in some genotypes. Alone, the intersib combinations revealed nothing further.

The pollen of 552 showed that S_5 was active in the stigma of S_1S_5 (Fig. 11), confirming a previous conclusion. In addition, the pollen of 552 showed S_5 to be active in the stigma of S_5S_8. With this new information we can return to the intersib results from family 3×5 (Fig. 2) and definitely state that S_5 was recessive in the pollen of S_1S_5, because this pollen was compatible on S_5S_5. Similarly the pollen of 554 showed that S_4 was active in the stigma of S_4S_8 (Fig. 11), and thus S_4 must have been recessive in the pollen of S_1S_4. The pollen of 554 itself had both S_4 and S_5 active.

Thus we know the activity of S_1, S_4, and S_5 in the four classes of 3×5 but have no precise evidence on the activity of S_8. Since S_4S_8 and S_5S_8 were mutually compatible in both directions, S_8 must have been recessive somewhere. As a tester for S_8 was never found, the question remains unsettled.

Family 3×2

The four classes of this family were discovered by sib combinations (Fig. 5). Plants 321 and 322 were mutually compatible in both directions and their pollen divided the family into two classes of 13 and seven plants each. Four plants of each class were then chosen and 63 combinations were made among them. This showed that 327 and 3210 belonged to two additional classes.

Meanwhile, the analysis of families 3×5 and 5×5 showed that parent 3 had the genotype S_1S_8. It followed that S_1 would be present in two classes of family 3×2. Accordingly, the pollen of 353, a tester for S_1, was applied to all plants of 3×2. The prediction was confirmed, and, at the same time, the remaining 12 plants were classified for S_1 (Fig. 5).

The interclass combinations of 3×2 were then arranged to form a compatible diagonal (Fig. 16), and the genotypes S_1S_2, S_2S_8, S_1S_3, and S_3S_8 were assigned on the assumption that parent 2 had the genotype S_2S_3. Had parent 2 been homozygous, only two classes would be expected. The intersib-compatibility pattern indicated that S_1, S_2, and S_3 were active wherever present. However, two compatible reactions between classes which had S_8 in common showed that this allele was somewhere recessive.

It will be recalled that the analysis of families 2×4 and 4×2 did not provide enough data for the assignment of genotypes. However, family 3×2 provided testers for the alleles of parent 2 so that it was possible to re-examine 2×4 for those alleles and, at the same time, to test part of the interpretation of family 3×2. The pollen of 241 and 245 was used on the four classes of 3×2 (Fig. 5) showing that S_2 was active in 241 pollen and S_3 active in 245 pollen. Similarly the pollen of 322 and 321 showed that S_2 was active in the stigmas of 241 and that S_3 was active in the stigmas of 245 (Fig. 10). It followed that the allele(s) of parent 4 were somewhere recessive in family 2×4.

Family 1×1

Parent plants 1 and 3 were mutually incompatible in both directions indicating a common allele. Analysis of family 3×5 showed that, of the alleles of parent 3, S_1 was higher in the dominance sequence than S_8. Therefore, it appeared probable that S_1 was the common allele. Accordingly, the eight plants of family 1×1 (produced by bud-selfing) were tested for S_1 by the stigmas of 3511 and 357 (Fig. 6). The prediction that family 1×1 would contain S_1 or S_8, or both, and the guess that it would be S_1, were confirmed when the testers divided the family into two classes. Intersib combinations further divided the class containing S_1. Genotypes were assigned on the assumption that plant 1 was S_1S_6. Sib combinations revealed that S_6 acted independently of S_1 in the stigma but was recessive to S_1 in the pollen.

The intersib combination 112×113 is interesting because it was partially compatible, although incompatibility was expected. At first, when five flowers were pollinated, three siliques developed and two failed. As usual with indecisive results, the pollination was repeated, special precautions being taken to ensure isolation. In practically all such cases the second pollination

3 × 2		12						28							15	38			
		7	13	16	17	18	19	2	4	5	8	9	14	20	10 11 15	1	3	6	12
12	7	−						−	−	−					−	+	+	+	
28	2	−	−	−	−	−	−	−	−	−	−	−	−	−	+ + +	+	+	+	+
	4	−						−	−	−					+	+	+	+	
	5	−						−	−	−					+		+	+	
13	10	−						+	+	+					−	−	−	−	
38	1	+	+	+	+	+	+	+	+	+	+	+	+	+	− − −	−	−	−	−
	3	+						+	+	+					−	−	−	−	
	6	+						+	+	+					−	−	−	−	
1(4)	353	−	−	−	−	−	−	+	+	+	+	+	+	+	− − −	+	+	+	+
1(6)	111	−						+	+	+					−	+	+	+	
2?	241	−						−	−	−					+	+	+	+	
3?	245	+						+	+	+					−	−	−	−	

5

1 × 1		11	16				66			15	
		6	1	2	8	9	3	4	7	3511	357
11	6	−	−	−	−	−	+	+	+	−	
1(6)	1	−	−	−	−	−	+	+	+	−	
	2	−		−	−	−	+	+	+	−	
	8	−		−	−			+		−	−
	9	−		−		−		+		−	−
66	3	+	−	±	−	−	−	−	−	+	
	4	+	−	−	−	−	−	−	−	+	
	7	+	−	−			−	−	−	+	+

6

2 × 4		27		29		37		39	
		2436	2444	2441	2443	2442		2437	2438
(27)	463	−	−	+	+	−		+	+
(29)	465	+	+	+	+	+		+	+

7

2 × 1		12		28		13	36		2?	3?	14	16
		2	5	3	8	9	1	4	241	2411	353	112
12	2	−	−	−	−	−	+	+	−	+	−	−
	5	−		−			+		−	+	−	−
	7						+		−	+	−	−
2(6)	3	−		−			+	+	−	+	+	+
	6	−			−	+	+		−	+	+	+
13	8								+	−	−	−
	9	−	−	+	+	−	−	−	+	−	−	−
3(6)	1	+	+	+	+	−	−	−	+	−	+	+
	4	+		+			−		+	−	+	+
66	113	+	+	±	−	+	−	−	+	+	+	−

8

2 × 6		2a		2b	3a			27	39	1a	1b
		4	5	1	2	3	6	2436	2437	632	634
2a	4	−	−	−	−	−	−	−	+		
	5	−			−	−		−	+		
2(b)	1	−	−	−	+	+	+	−	+		+
	7							−		+	
3a	2	−	−	+	−	−	−	+	−		
	3	−			−			+	−		
	6	−			−			+	−		
	8							+		−	
	9							+		−	
2(7)	2436	−		−	+	+	+				
1a	632	−	−	+	−	−	−				
b9	465			−							

9

FIG. 5. Sib combinations in family 3×2 and the reaction of pollen testers for S_1, S_2, and S_3 toward this family.

FIG. 6. Sib combinations in family 1×1 and the reaction of stigma testers for S_1 toward this family.

FIG. 7. Demonstration of four classes in family 2×4.

FIG. 8. Sib combinations of family 2×1; reaction of stigma testers for S_1, S_2, S_3, and S_6, and of pollen testers for S_6 toward this family.

FIG. 9. Sib combinations of family 2×6 and the reaction of pollen and stigma testers for S_2, S_3, S_a, and S_b toward this family.

conclusively decided the point but in this instance, and with two further pollinations at roughly 1 week intervals, the results remained consistently inconclusive. In all, 12 siliques developed and eight failed. Similar results with *Iberis* (1) were thought to be due to modifying genes.

Families 2×1 and 1×2

Analysis of family 3×2 established that the genotype of parent 2 was S_2S_3. Testers for both alleles were available in the families of 3×2, 2×4, and 4×2. Similarly, family 1×1 provided testers for S_1 and S_6 of parent 1. Testers for S_1 were also available in family 3×5. Thus it was not only predicted that family 2×1 and its reciprocal would contain the genotypes S_1S_2, S_2S_6, S_1S_3, and S_3S_6, but testers were available for all alleles. In addition, the phenotypes of S_1S_2 and S_1S_3 were known from family 3×2 and should be the same in families 2×1 and 1×2.

The two families were examined for S_2 and S_3 by means of females from family 2×4, and for S_1 by females from family 3×5. The four classes were found in the nine plants of 2×1 (Fig. 8) but one was missing from the nine plants of 1×2 (Fig. 12). Sib combinations in 2×1 independently revealed the same four classes (Fig. 8). Interfamily combinations between numbers of 2×1 and 1×2 behaved as expected (Fig. 14).

The stigma of 112 showed that allele S_6 was recessive in the pollen of S_2S_6 and S_3S_6, and the pollen of 113 showed S_6 to be active in the stigmas of S_2S_6 and S_3S_6.

Plant 213 was exceptional in that it was partially compatible with the pollen of 113 although incompatibility was expected. Of 10 flowers pollinated, five siliques developed fully, two developed slightly, and three failed. This partial compatibility is similar to that encountered in family 1×1 and involves the same pollen plant. If modifying genes are indeed responsible, the data suggest that the modifiers have to be expressed in both the pollen and the stigmas concerned. Possibly some complementary reaction is weakening the incompatibility.

Family 3×1

This single plant was the product of pseudocompatibility. Twenty-two flowers of parent 3 were pollinated by parent 1 but only two siliques, each with one seed, developed (Table I). Plant 311 was incompatible with 353 in both directions; thus it possessed S_1. The pollen of 311 was compatible on 113 but its stigma rejected the pollen of 113 indicating that S_6 was the other allele. This definitely proved that 311 was a product of pseudocompatibility and not the result of contamination, because S_6 was found only in parent 1.

Family 3×3

Another single plant, the product of bud pollination, like 311 was incompatible with 353 in both directions and therefore had S_1. This was unfortunate because a plant homozygous for S_6 would have been valuable for the analysis

of allele activity. In the entire study, no activity could be ascribed to S_8 and therefore it could not be determined whether 331 was S_1S_1 or S_1S_8. Unlike 311, 331 was compatible with 113 in both directions, as expected.

Families 4×1 and 1×4

Family 4×1 contained two plants; its reciprocal, one. The genotype of parent 1 was S_1S_6; that of parent 4 was unknown. The pollen of all three plants was rejected by the stigma of 356 showing that all had S_1. The three plants were mutually incompatible.

Further analysis was delayed until results from the hybrid family 4×6 showed that parent 4 had the genotype S_7S_9. Family 4×6 also provided a tester for S_7. The pollen of 463 (S_7S_b) was compatible on 412 (S_1S_9) and incompatible with 411 (S_1S_7) and 141 (S_1S_7).

In the meantime, the three plants were used with no success in an attempt to reveal further classes in family 2×4 (Fig. 10). Additional interfamily crosses showed that 141 and 411 accepted the pollen of 241 (S_2S_7), 245 (S_3S_7), 117 (S_6S_6), 6311 (S_bS_9), 461 (S_aS_7), and 465 (S_bS_9), but rejected the pollen of 353 (S_1S_4).

Interfamily Combinations

Analysis of incompatibility in 12 families of *L. densipila* revealed the presence of at least 18 possible genotypes among the progeny. However, at this time, nothing was known about the alleles of parent 4 so that additional genotypes could be expected in families 2×4, 4×2, 1×4, and 4×1. In fact, evidence later obtained from family 4×6 showed that probably 21 and possibly 22 genotypes were present in the 12 families. It was desirable to intercross as many genotypes as possible, both to check previous conclusions and to provide further evidence that the nine proposed alleles actually were different from each other. Many of the data from this experiment have been considered during the analysis of individual families. However, Fig. 10 brings them together in a more digestible form.

To provide a good chance of representing the four possible genotypes, eight plants of family 2×4 were entered as females and two as males. Then genotype S_1S_1 was not entered because S_1 was already represented by seven other genotypes. The genotypes of family 2×1 were entered only as males. Unfortunately, the genotypes S_4S_5 and S_5S_5, from family 5×5, were no longer available for the experiment.

It was known that S_1, S_2, S_3, S_4, S_5, and S_9 were active in stigmas of the genotypes represented and that S_1, S_2, S_3, and S_6 were active in the pollen. The behavior of these alleles was exactly as expected. No new information on allele activity was forthcoming.

Hybrid Families

The search for more revealing data on the activity of S_9 from parent 3 and S_7 and S_9 from parent 4 was directed toward three hybrid families between one plant of *L. lescurii* (parent 6) and three of *L. densipila* (parents 2, 3, and 4).

Inter-family		2?				3?				i4	i5	48	58	i2	28	i3	38	i6	66
		241	242	243	2415	245	246	2411	2416	353	358	351	354	327	322	3210	321	112	113 117
2?	241	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	
3?	245	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−	+	
i(4)	353	+	+	+	+	+	+	+		−	−	+	+	−	+	−	+	−	+ +
i(5)	358	+								−	−	+	+	−	+	−	+	−	
48	351	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+
58	354	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+
i2	327										−	+	+	−	−	−	+		
28	322	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+
i3	3210									−	−	+	+	−	+	−	−		
38	321	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−	+	+
i(6)	111	+				+		+		−	−	+	+	−	+	−	+	−	+ +
66	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	− −
i2	212	−	−	−	−	+	+	+	+	−	−	+	+	−	−	−	+	−	+
2(6)	213	−						+		+	+	+	+	−	−	+	+	+	+
i3	219	+						−		−	−	+	+	−	+	−	−	−	+
3(6)	211	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−	+	+
i(7)	411	+	+	+	+	+	+	+	+										
	141	+	+	+	+	+	+	+	+										
i9	412	+	+	+	+	+	+	+	+										

10

5 x 5		i4	i5	46	36
		353	358	351	354
45	551	−	−	−	−
	553	−	−	−	−
	554	−	−	−	−
55	552	+	−	+	−

11

1 x 2		27	39	i4	i5
		2436	2438	353	357
i2	1	−	+	−	−
	4	−	+	−	
	6	−	+		−
i3	2	+	−	−	−
	3	+	−		−
	7	+	−	−	
	8	+	−	−	
36	5	+	−	+	
	9	+	−	+	

12

6 x 3		ia							a8					ib	b8	i4
		1	2	3	5	10	13	14	6	7	8	9	12	4	11	3516
ia	1	−							−					−		−
	2	−	−	−	−				−	−	−			−		−
	3			−					−					−		−
	5	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
	10															−
	13															−
	14															−
a8	6	−	−	−	−				−	−	−	−		+	+	+
	7		−		−				−	−	−			+		+
	8								−	−	−			+		+
	9				−											+
	12				−											+
i(b)	4	−	−	−	−				+	+	+			−	+	−
b8	11				+				+					−	−	+
i(4)	353	−	−	−	−				+	+	+			−		

13

2x1 & 1x2		i2	i3	36	i2	26
		121	123	125	212	213
i2	212	−	−	+		
2(6)	213	−	+	+		
i3	219	−	−			
3(6)	211	+	−	−		
i2	121				−	−
i3	122				−	+

14

4 x 6		a7	b9	b7	ia	ib
		1 4	5	3	632	634
a(7)	1	−	+	+	−	+
	4	− −		+	−	
b9	5	+ +	−	−		−
b7	3	− −	−	−	+	−
ia	632	− −		+	−	−
i(b)	634	+ +		+	−	−

15

FIG. 10. Interfamily combinations of *L. densipila*.

FIG. 11. Reaction of the pollen of family 5×5 toward the class representatives of family 3×5.

FIG. 12. Classification of family 1×2 by means of stigma testers for S_1, S_2, and S_3.

FIG. 13. Sib combinations of family 6×3 and the reaction of pollen and stigma testers for S_1 toward this family.

FIG. 14. Combinations between family 2×1 and 1×2.

FIG. 15. Sib combinations of family 4×6 and reactions of testers for S_a and S_b toward this family.

The unimpaired self-incompatibility of these hybrid families is interesting in view of the natural hybrid populations between these two species which Rollins (5) has described from the wild. The morphological variability in the natural hybrid populations is intermediate between that of the two parents, and shows no skewness toward either parent. Possibly enforced heterozygosity, due to self-incompatibility, is one of the factors responsible for the variation pattern of the natural hybrids.

Family 6×3

The largest and only complete hybrid family studied contained 14 plants. It was predicted that S_1 from parent 3 would be present. This was confirmed when the stigma of 3516 divided the family into two groups. Further analysis utilized sib combinations with the pollen of 635 revealing two additional classes (Fig. 13).

The compatible diagonal was constructed and the genotypes S_1S_a, S_aS_8, S_1S_b, S_bS_8 were assigned to the classes (Fig. 16). (Alleles of parent 6 were designated S_a and S_b to show they were derived from a different species than those of the numbered series.) Sib combinations showed that S_1 and S_a were active in pollen and stigmas wherever present. Similarly S_b was active in the stigma of S_1S_b and the pollen of S_bS_8. Three compatible combinations between classes with common alleles showed that S_b was recessive in the pollen of S_1S_b, or the stigma of S_bS_8, or in both.

No sib incompatibility could be attributed to S_8. Similarly, S_8 was not active in interfamily combinations: 637 (S_aS_8) and 6311 (S_bS_8) were compatible with 351 (S_4S_8) and 322 (S_2S_8) in both directions; 637 was compatible with 354 (S_5S_8) and 321 (S_3S_8) in both directions and the pollen of 6311 was compatible on 354.

Family 2×6

The genotypes S_2S_a, S_2S_b, S_3S_a, and S_3S_b were predicted for this family, and testers were available for all alleles. The first six plants were tested for S_2 and S_3 with the stigmas of 2436 and 2437, and the pollen of 632 was used to test for S_a. Three genotypes were revealed (Fig. 9), but the fourth was not found when three remaining plants were tested for S_2 and S_a with the stigmas of 2436 and 632.

Sib and interfamily pollinations agree in showing that S_2 and S_a were active wherever present. S_3 was active in the pollen of S_3S_a and probably also in the stigma but this was not shown. The activity of S_b in S_2S_b was determined by a tester from family 4×6.

Family 4×6

This small but important family contained four plants and three genotypes. Plants 632 and 634, used as testers for S_a and S_b, revealed two classes. Sib combinations revealed a third class, thus demonstrating for the first time that parent 4 had been heterozygous (Fig. 15).

It is somewhat ironical that the last family studied, a small family analyzed by but 25 pollinations, should provide information not forthcoming from families 2×4 and 4×2 where over 360 pollinations were carried out. Only two intra-incompatible and inter-compatible classes were found in family 2×4. Such a pattern results from the activity of two alleles but the analysis of families 2×1, 1×2, and 3×2 showed that these two alleles were derived from parent 2. No activity of the alleles from parent 4 was detected in families 2×4 and 4×2. However, the results from family 4×6 showed that parent 4 was heterozygous S_7S_9.

Analysis of family 4×6 showed that S_b was active in both the pollen and stigmas of S_bS_7 and S_bS_9. Activity of S_7 was demonstrated in the pollen of S_bS_7 and in the stigma of S_aS_7. The pollen of 461 was compatible on 411 and 141 (S_1S_7) showing that S_7 was recessive in the pollen of S_aS_7. The pollen of 117 (S_6S_8) was compatible on 461, 463, and 465.

Further Analysis of Family 2×4

Discovery of the activity of S_7 in the pollen of 463 permitted family 2×4 to be reinvestigated. Seven plants were tested and four genotypes were revealed (Fig. 7). S_7 proved to be active in stigmas of S_2S_7 and S_3S_7. Therefore, it must have been recessive in the pollen of these genotypes.

Nowhere in this study could any activity be attributed to S_9. However, it is conceivable that S_9 was active in the pollen of 465 (S_bS_9) since S_b was low in the dominance sequence in pollen. Accordingly, pollen of 465 was used on 22 plants of 2×4 and six plants of 4×2 with full compatibility in each cross. The plants of 2×4 used in this experiment, in addition to the seven given in Fig. 7, were 241, 242, 243, 244, 2412, 2415, 2418, 2423, 2425, and 2440 having S_2; 245, 246, 2411, 2416, and 2433 having S_3. The plants of 4×2 used were 4213, 4223, 4224, and 4226 having S_2; 426 and 4214 with S_3.

Identity of Alleles

It is possible to obtain 12 *S*-alleles from six parents, but the incompatibility between parents 1 and 3 reduced this maximum to 11. To facilitate analysis, the maximum number was assumed and *S*-genotypes were assigned to the parents as follows:

Parent	1	2	3	4	5	6
Genotype	1.6	2.3	1.8	7.9	4.5	*a.b*

Because the progeny analysis yielded no evidence that any two of these alleles were identical, the maximum remains at 11. However, this does not prove that 11 different alleles were present. It must be shown that each is distinct from all others. For example, it is necessary to show that S_1 is not S_2, nor S_3, nor S_4, and so on. With 11 alleles there are 55 such propositions to be verified.

Five sorts of data were accepted as categorical proof that the two alleles in question were distinct. These are: (*a*) if the two alleles segregate as alternates from the one parent; (*b*) if the two alleles are both active in the same sex of one plant; (*c*) if two plants, each with one of the two alleles active in

the appropriate sex, are compatible; (*d*) if any observed sib-compatibility pattern is impossible when the two alleles in question are identical; and (*e*) if one of the two alleles is recessive to a third, while the other is not.

Forty-nine propositions were established from the five types of data. The formal proofs will not be listed; the data are already given. It was not shown that S_7 was not S_4 nor S_5, and that S_9 was not S_4, nor S_5, nor S_6, nor S_8. Thus, if S_7 and S_9 are removed, each of the remaining nine alleles is unique.

Proofs of three additional propositions may be based on the probability that S_7 and S_9 were present in the eight plants of family 2×4, which were entered as females in the combinations shown in Fig. 10. Of necessity one allele was represented and there is only one chance in 256 that the other was not also present. Therefore, S_6 is probably not S_9, for if it were, since S_6 was active in the stigmas of S_2S_6 and S_3S_6 in family 2×1, the pollen of 113 (S_6S_6) should have been incompatible on some of the eight testers of family 2×4, but it was not. Likewise, S_5 is probably not S_7, because S_7 was shown to be active in the stigmas of family 2×4, yet the pollen of 354 (S_5 active) was compatible on the eight testers. The probability is even stronger that S_4 is not S_7 because the pollen of 351 (S_4 active) was compatible on the eight testers as well as on 244, 247, 248, and 249.

Thus, only S_9 was not established. It may have been the same as S_4 or S_5 or S_8. Therefore, the minimum number of *S*-alleles in the material was 10.

Segregation of Alleles

The populations are too small to permit satisfactory analysis for random recombination of the *S*-alleles, but the segregation of alternate alleles from each parent can be obtained by pooling data from applicable families. A 1:1 ratio is expected.

Five families gave an observed ratio of 21 S_1:16 S_6 from parent 1 ($\chi^2=0.68$; $P>.30$). Five families gave 62 S_2: 46 S_3 from parent 2 ($\chi^2=2.38$; $P>.10$). The deficiency of S_3 is nevertheless considerable. Part of it may be due to associated viability factors, because the first 35 plants of family 2×4 were numbered in order as they came into flower, and Fig. 1 shows the preponderance of early flowering plants with S_2.

Three families gave 27 S_1:27 S_8 from parent 3. The seven completely analyzed plants of family 2×4 and family 4×6 gave 6 S_7:5 S_9 from parent 4. Two families gave 15 S_4:13 S_5 from parent 5.

The segregation from parent 6 deviated significantly from a 1:1 ratio. Three families gave 21 S_a:6 S_b ($\chi^2=8.4$; $P<.01$). Viability factors, as in family 2×4, may be responsible. Under the same cultural conditions, part of family 2×4 suffered 28% mortality, family 2×6 suffered 37% and family 6×3, 58%. A sample of 300 or more pollen grains of each parent was examined in acetocarmine. The percentage of morphologically good pollen ranged from 91 for parent 3 to 99 for parent 6. Thus it is unlikely that the observed deviation in the segregation of *S*-alleles from parent 6 was due to meiotic irregularity.

Allele Action

Sixty-five genotypes are possible with the 11 proposed alleles, and 35 were present in the material. The phenotypes of 17 genotypes are known completely, 16 are known in part, but the phenotypes of parents 2 and 6 are completely unknown.

Only two types of gene interaction, independence and dominance, were found in heterozygotes. Fig. 17 shows the established relationships. Of the nine alleles which act independently of each other in the stigmas, S_1, S_2, S_3, and S_a are dominant over S_4, S_5, S_6, S_7, and S_b in the pollen. This suggests that the nine alleles belong to the same dominance level in the stigmas, but to at least two levels in the pollen.

The question immediately arises whether the dominance sequence is indeed linear as just stated. For example, since it was shown that S_1 and S_2 are both active in the pollen of S_1S_2, and that S_1 is dominant over S_4 in the pollen of S_1S_4, does it follow that S_2 will be dominant over S_4 in the pollen of S_2S_4?

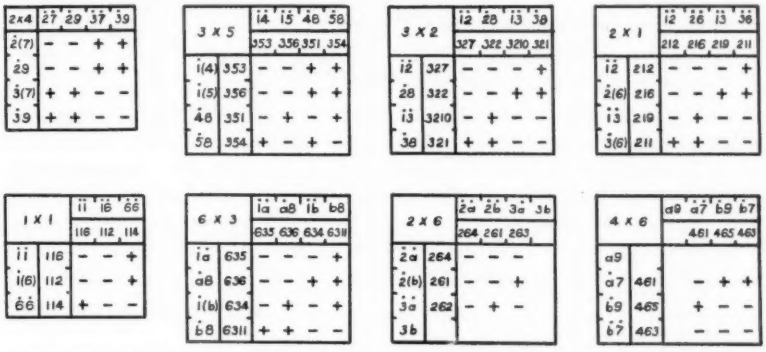

FIG. 16. Compatibility relationships of class representatives of the principal families arranged to form compatible diagonals.

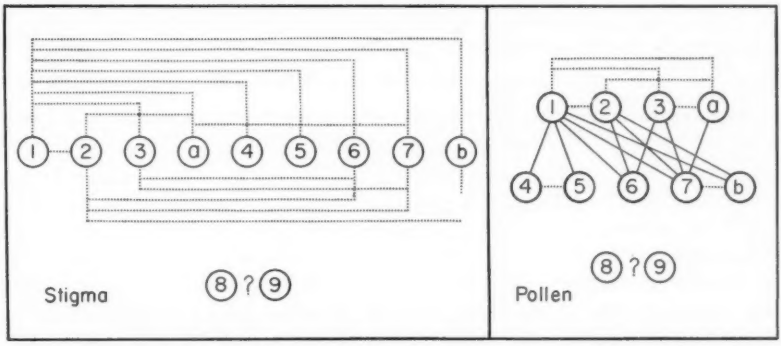

FIG. 17. Established dominance relations of *Lesquerella* incompatibility alleles. Each allele is represented by a numbered or lettered circle. Dotted lines denote independence of action in heterozygotes; solid lines, dominance of upper over lower.

If so, then many unknown phenotypes can be predicted. However, there is no *a priori* reason to assume a linear dominance sequence except that almost all reliable data from other multiple allelic, sporophytic systems do fit a linear pattern. The exceptions are S_5 in *Brassica campestris* (2), possibly S_3S_4 in *Iberis*, second series (1), possibly S_1S_2 in the reinterpretation of Sears' *Brassica oleracea* data (7) and definitely the relation in the style of *B. oleracea* where $S_f = S_a$ and $S_a = S_e$ but $S_f > S_e$ (incompletely dominant ?) (8).

In *Lesquerella*, the activities of S_8 and S_7 are almost completely unknown. They are somewhere recessive, but their precise activity could not be established because we lacked testers in which they were active. Likewise, S_7 was phenotypically unknown until it was found active in the pollen of S_bS_7. The activity of S_7 was not found earlier because it was nowhere else in combination with an allele of the lower dominance level. This argument cannot account for the still unknown activity of S_8 and S_9 because S_8 was present in the genotypes S_4S_8, S_5S_8, and S_bS_8, and S_9 was present in S_bS_9. Therefore, if the dominance relations follow a linear order, S_8 and S_9 must belong to a third and lower dominance level.

Acknowledgments

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THE pH FACTOR IN THE METABOLISM OF FUSARIUM MONILIFORME SHELDON ON SUCROSE[1]

KAISER NAGUIB

Abstract

A study has been made of the effects of varying the pH value of the culture media on sucrose inversion and absorption, on respiration, and on growth of *Fusarium moniliforme* Sheldon. The study was carried out on 1-week-old fungal mats that were then subjected to experimentation for 48 hours. Sucrose inversion was almost unaffected by pH values higher than 5.4 but was inhibited at pH 4.0. Sugar uptake was influenced more by lowering than by raising pH beyond 5.4. Carbon dioxide output per unit dry weight of mycelial mat was lowest at the pH optimum for growth, as measured by mat dry weight. At the same optimum pH there was also the highest rate of sucrose inversion and uptake. It seemed, therefore, that the effect of pH on growth was the additive effects on the different enzymatic processes operating in the metabolism of the fungus. It is also suggested that the external pH of the medium might act by the invasion of hydrogen or hydroxyl ions into the mycelial cells, thus changing the internal pH.

Introduction

The importance of pH as a physiological factor in the metabolism of fungi in culture media has long been claimed (Wolpert (25), Lilly and Barnett (11), Emerson and Cantino (7), and Apparao (1)). It was desired in the present investigation to find out the effects of this factor on the metabolism of *Fusarium moniliforme* on sucrose. This work was conducted as a part of a detailed study for the elucidation of the effects of the different physiological and environmental conditions on the carbohydrate metabolism of this fungus. Most of the previous workers, e.g. Scott (21), White (24), and Pritham and Anderson (14), dealt with unbuffered media. It was thought advisable here to use controlled pH conditions.

Materials and Methods

The Organism

The fungus used in the present investigation was *Fusarium moniliforme* Sheldon isolated from the Egyptian soil near Cairo and identified by Dr. W. L. Gordon of the University of Manitoba, Canada, to whom the author is greatly indebted.

Cultural Conditions and Composition of the Media

The fungus was maintained on Dox's agar slopes and subcultured at intervals of 14 days. Dox's agar is made up with sucrose, sodium nitrate, potassium dihydrogen phosphate, potassium chloride, magnesium sulphate, and ferrous sulphate. The fungus was first grown on liquid Dox's medium in 250 ml. Erlenmeyer flasks. Inoculation was carried out by a spore suspension made up from 1-week-old slant cultures on Dox's agar. The culture flasks were then incubated at 25° C. until 1-week-old fungal mats were formed. These

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Contribution from the Botany Department, Faculty of Science, Cairo University, Giza, Egypt.

mats were then aseptically freed of the old media and washed three times by carefully passing sterilized distilled water below the felts and decanting the washings each time as far as possible. The fungal mats were then fed with fresh media of the following basal constitution (g./liter): sodium nitrate, 2.0; potassium dihydrogen phosphate, 1.0; magnesium sulphate, 0.5; potassium chloride, 0.5; ferrous sulphate, 0.01; buffered with 11.8 succinic acid and 14.2 disodium monohydrogen phosphate, and then made up with distilled water. The basal medium was divided into four parts. By appropriate addition of concentrated hydrochloric acid or sodium hydroxide to the different parts, four different media with pH values 4.0, 5.4, 6.8, and 8.4 were prepared. The culture media were sterilized and sucrose added aseptically at 40 g. per liter. The washed fungal mats, under sterile conditions, were fed in four replicates with 50 ml. each of the appropriate medium. Estimations of the initial sucrose concentration and pH values were carried out directly after addition of the media to separate duplicate samples. This precaution was necessary to allow for any change in the media when they came in contact with the lower mycelial surfaces.

The culture flasks were freed of any carbon dioxide present and incubated at 25° C. The experiment was run for 48 hours, during which time measurement of the carbon dioxide given off and estimation of the reducing sugars (direct reducing value) as well as of the total residual sugar (total reducing value) were carried out at 24-hour intervals. The dry weights of mycelial mats were also obtained after they were separated from their media by dry filtration followed by drying at 60° C. to constant weight.

Methods of Analysis

For the removal of carbon dioxide originally present and the estimation of that given off after the two intervals specified above, the culture flasks were stoppered with sterilized rubber stoppers fitted with inlet and outlet tubes and then adjusted into an apparatus designed for the purpose and given in a previous communication by Saïd and Naguib (19).

Estimation of reducing sugars in the media was carried out by a modified Schaffer-Hartmann's method as given by Saïd and Naguib (18) in a previous work on the same fungus. This gives an estimate of the direct reducing value. The total reducing value was determined by the same method after acid hydrolysis. This denotes the amount of residual sugar in the medium.

Results

Analysis of the Culture Media

Sugar concentration dropped in all the media at the different pH values (Fig. 1), the biggest drop being at pH 5.4 and the smallest at 4.0. Rise in pH beyond 5.7 induced a smaller drop in sugar concentration.

Reducing sugars appeared in the culture media after 24 and 48 hours, showing inversion of sucrose by the mycelial cells. This also showed that rate of inversion exceeded rate of absorption of inversion products so that the latter appeared and accumulated in the external media. Sucrose inversion

by *Fusarium moniliforme* has been studied in a previous communication (Saïd and Naguib (18)). It has also been shown that inversion was affected by invertase centers located at the plasmatic surfaces of the fungal cells. Several workers have also recorded sucrose inversion by other types of plant cells, e.g. Saïd (17), Burström (3), and Forsyth and Webley (8).

The rate of accumulation of inversion products was slightly affected by change of pH during the first 24 hours, but later on there was higher accumulation in media with pH 4.0 than in the other media.

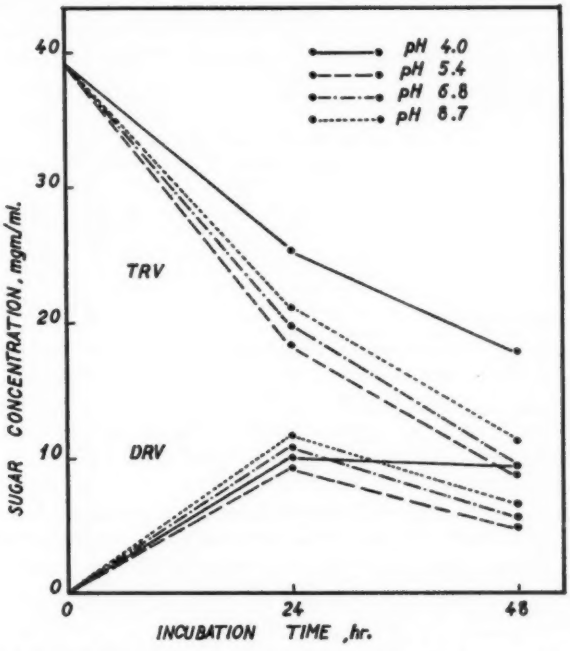

FIG. 1. Drifts in the direct (D.R.V.) and total reducing values (T.R.V.) of the sucrose media at different pH values.

Sucrose Inversion and Sugar Uptake

Assuming that sucrose was inverted before absorption, the sum of the amount of reducing sugars in the medium and the amount of sucrose absorbed would give an estimate of the total amount of sucrose inverted. Amounts of sugar uptake and total sucrose inversion in the different experimental media are given in Table I.

Sucrose inversion by the mycelial mats was almost unaffected by change of pH from 5.4 to 8.7 but at pH 4.0 there was a well-marked suppression in the rate of inversion. It is worthy of mention here that Charles and Farrell (4), in their work on dextranase from *Penicillium lilacinum*, have shown that

the enzyme activity declined gradually above 6.5 while below 4.5 there was a sharp decrease to zero at pH 3.0; between 4.7 and 6.5 the enzyme activity was essentially independent of pH.

Sugar uptake was maximum at pH 5.4 with gradual decrease with increase of pH. Lowering pH to 4.0 markedly inhibited sugar absorption. The presence of relatively large amounts of reducing sugars in the external media showed that suppression of sugar uptake by increase or decrease of pH beyond 5.4 was independent of rate of inversion.

TABLE I

SUCROSE INVERSION AND SUGAR UPTAKE
(mg./ml.)

pH of medium	After 24 hours		After 48 hours	
	Sucrose inversion	Sugar uptake	Sucrose inversion	Sugar uptake
4.0	23.69	13.54	30.74	21.02
5.4	29.80	20.45	34.54	29.66
6.8	29.31	19.01	34.78	29.38
8.7	29.02	17.57	34.13	27.65

Carbon Dioxide Output

From Table II it can be easily noted that maximum carbon dioxide output was at pH 5.4 in the first as well as the second 24-hour period. There was a drop in the rate of carbon dioxide output when the pH was lowered or raised. At pH 4.0 there was more carbon dioxide given off in the first than in the second 24-hour period, while at pH values higher than 5.4, the rate of carbon dioxide output increased with time.

If amounts of carbon dioxide produced are calculated per gram dry weight of mycelial mat (Table II), one finds that minimum amounts were given off at pH 5.4. Rise or drop of pH induced higher output per gram dry weight.

Dry Weights of the Mycelial Mats and the Economic Coefficient

The rate of growth as indicated by the dry weights of the mycelial mats (Table III) was maximum at pH 5.4 with sharp decline at the lower pH value tested and gradual decrease with rise of pH. It could also be noted that

TABLE II

CARBON DIOXIDE OUTPUT

pH of medium	In 1st 24 hr. (mg./mat)	In 2nd 24 hr. (mg./mat)	Total in 48 hr.	
			mg./mat	mg./g. dry wt.*
4.0	301.8	282.5	584.3	1873
5.4	322.1	382.8	704.9	1408
6.8	291.3	346.7	638.0	1701
8.7	273.7	313.3	587.0	1672

* The dry weight here was taken as the average weight during the experimental period.

the rate of growth was influenced to a much greater extent by change of pH than was sucrose inversion or uptake. This observation might show that the effect of pH on growth was an additive result of its effects on several metabolic processes.

The economic coefficient, which denotes the increase in grams in dry weight produced per 100 g. sugar consumed, was also calculated and included in Table III. At the optimum pH for growth there was also maximum economic coefficient, the sugar consumed being mainly directed towards the building up of cellular substance rather than towards respiration or accumulation of intermediate products in the external media. This would agree nicely with the figures representing amounts of carbon dioxide given off per unit dry weight, where least output took place at the optimum pH. On the acid side of the optimum pH value, there was a sharp drop in the economic coefficient while it declined much less steeply with rise of pH.

TABLE III

DRY WEIGHTS OF THE MYCELIAL MATS AND THE ECONOMIC COEFFICIENT

pH of medium	Dry weight (mg./mat)			E.C., g. dry wt./* 100 g. sugar
	Initial	After 24 hr.	After 48 hr.	
4.0	275	299	349	7.0
5.4		492	722	30.1
6.8		377	476	13.7
8.7		353	427	11.0

* The dry weight here was taken as the increase in the dry weight during the experimental period.

Discussion

The fact that sucrose undergoes inversion by means of invertase centers located at the plasmatic surfaces of mycelial cells when it comes in contact with fungal mats of *Fusarium moniliforme* has been dealt with in a previous communication (Saïd and Naguib (18)). In the present work it was found that these enzyme centers were affected by the pH of the medium on the acidic rather than on the alkaline side.

Sugar uptake was also affected by change of pH with an optimum around 5.4 and a marked drop on the acidic side. The drop in sugar uptake at low pH values might be attributed to a loss of phosphate ions required for sugar phosphorylation. Scott (20), in his work with *Chlorella*, found that at low pH values phosphate ions were lost. He attributed this loss to instability of phosphate-containing compounds in the cells in acid media. The fact that decrease in phosphate content is accompanied by a drop in sugar uptake has been previously recorded by Hockenhull and co-workers (10) in their work with *Streptomyces griseus*. Di Marco and co-workers (5) showed that addition of phosphate to growing cultures of *Streptomyces aureofaciens* enhanced the rate of sucrose consumption.

It was further found that the drop in sugar uptake at low pH values was accompanied by a drop in the rate of growth and in total carbon dioxide

output. Hockenhull and co-workers (10) have also shown that respiration measured by the rate of glucose oxidation was dependent on pH value.

Sucrose inversion and sugar uptake were found to be lower in the second than in the first 24-hour period. This observation has been noted in previous work on the same organism. Whether or not this phenomenon was due to the presence of metabolic staling products that were excreted in the medium and were of inhibitory nature to enzymatic activity, to aging of the mycelial mats, or to other factors needs clarification in further work.

It could also be found that the extent to which the absorbed sugar was assimilated or dissimilated was dependent on the pH of the medium. At pH 5.4 there was a higher rate of building up of cellular substances accompanied by a lower rate of carbon dioxide output per unit dry weight of mycelial mat than at pH values below or above. The limitation of the pH value of the nutritive media in technical applications would be, in the light of the present findings, to adjust the metabolic activities of the microorganism towards maximum production of the substance required (Dimond and Peltier (6), Foster (9)).

The optimum pH value for sucrose inversion, sugar uptake, and carbon dioxide output was found to coincide with that for the dry weight increase, a fact that would suggest that the effect of pH on the rate of growth took place through affecting the different enzymatic processes going on at the same time. Lilly and Barnett (12) compared the effect of pH on urease activity (Van Slyke (23)) with its effect on growth (Ryan and co-workers (16)) and came to a similar conclusion.

The manifestation of the effects of varying the pH of the external media of *Fusarium moniliforme* on sucrose inversion and uptake, on respiration, and on growth might be explained on the assumption that the internal pH of the mycelial cells might change consequently. With respect to this, it might be mentioned that Bünning (2) found that the internal pH of *Aspergillus niger* changed with change of pH of the external medium. This conclusion is analogous to that arrived at by Rennerfelt (15) in his study of the ion absorption by *Aspergillus niger*. He found that the internal ion concentration of the fungal cells changed with change of external ion concentration in the culture medium. Steemann Nielsen (22), in his work with *Chlorella*, suggested the invasion of H^+ or OH^- ions into the cells according to whether the external pH of the medium was low or high respectively. Finally, it is interesting to note that Mahdihassan (13) could determine the internal pH of cells of *Fusarium lini* B to be 6.0–6.1, a range that is quite near the optimal region for *F. moniliforme* found in the present work.

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AN ASSOCIATION BETWEEN VARIABILITY IN OVULE DEVELOPMENT WITHIN OVARIES AND SELF-INCOMPATIBILITY IN LOTUS (LEGUMINOSAE)[1]

J. S. BUBAR

Abstract

Ovules within each ovary of self-fertile *Lotus* species develop uniformly while those within each ovary of self-sterile species vary considerably in rate of development. All ovules in self-fertile species may be fertilized while less than one-half of those in self-sterile species are generally fertilized. Time of pollination is determined by the time when the stigmatic membrane ruptures in self-fertile species, by the time of rupturing by pollinating insects in self-sterile species. Higher numbers of ovules per ovary compensate for the proportion not fertilized in self-sterile species. The relationships of self-sterility to self-incompatibility in this genus, the evolution of this mechanism, and evidence that similar mechanisms are found in other legumes are discussed.

Introduction

Hansen (7) described normal ovule development in *Lotus corniculatus* L. for individual ovules but he did not compare the rate of development of different ovules within the same ovary. He found an average of 59 ovules per ovary and of 19 seeds per pod. The writer observed, in some preliminary studies of self-incompatibility which led to the investigation reported in the paper, that many ovules in each ovary of a number of self-incompatible *Lotus* species do not develop into viable seeds following cross-pollination. He also observed that all ovules develop into seeds in some naturally self-pollinated species such as *L. corniculatus* var. *japonicus* Regel and *L. angustissimus* L. These data are incorporated into Table II.

Materials and Methods

Sources of plant materials are listed in Table I. These materials were grown in field nurseries during the summers of 1954, 1955, and 1956 and in a greenhouse during the intervening winters. Numbers of ovules per ovary and of seeds per pod were determined from plants in field nurseries. The numbers presented are averages from 10 pods and 10 ovaries collected on a representative plant at one time. Self-fertility and the behavior of the stigmatic membrane was studied on plants in the greenhouse in the wintertime. No pollinating insects were present in this greenhouse. Self-fertility was recorded for undisturbed flowers (under column headed "without manipulation" in Table II) and for flowers which were manipulated to induce self-pollination. A piece of fine sandpaper was held against the pore in the

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tip of the keel and the stigma was forced through the pore against the sandpaper and allowed to return into the mass of pollen inside the keel. Fresh sandpaper was used for each different plant manipulated to ensure that no cross-pollination occurred.

TABLE I

PLANT MATERIALS

Collection	Source
L. corniculatus L.	Mr. A. Charbonneau, Agronome, Joliette, Que., from old stand near Ste. Melanie, Que. Seed originated in New York State
L. tenuis Wald. et Kit.	Forage Crops Division, Central Experimental Farm, Ottawa
L. uliginosus Schkuhr.	Columbia variety from Astoria, Oregon
L. weilleri Maire	Forage Crops Division, Central Experimental Farm, Ottawa, Accession 1893-3836
L. suaveolens Pers.	Forage Crops Division, Central Experimental Farm, Ottawa
L. jacobaeus L.	Forage Crops Division, Central Experimental Farm, Ottawa, Accession 1893-4538
L. corniculatus var. *japonicus* Regel.	Professor Isawo Hirayoshi, Kyoto University, Kyoto, Japan, collected on a river bank near Gifu
L. angustissimus L.	Forage Crops Division, Central Experimental Farm, Ottawa, Accession 1893-3576
L. hispidus Desf.	Forage Crops Division, Central Experimental Farm, Ottawa, Accession 1893-3950
L. palustris Willd.	Forage Crops Division, Central Experimental Farm, Ottawa, Accession 1893-3575
L. filicaulis Durien.	Dr. P. Henson, U.S.D.A., Beltsville, Md., U.S.A. Accession P.I.51864
L. parviflorus Desf.	Forage Crops Division, Central Experimental Farm, Ottawa
L. divaricatus Boiss.	Dr. P. Henson, U.S.D.A., Beltsville, Md., U.S.A. Accession P.I.109314
L. perigrinus L.	Forage Crops Division, Central Experimental Farm, Ottawa
L. tetragonolobus L.	Dr. P. Henson, U.S.D.A., Beltsville, Md., U.S.A. Accession P.I.67217
Hosackia Dougl. sp.	Dr. V. C. Brink, University of British Columbia, Vancouver, B.C.

Ovule development was studied from serial sections of entire ovaries cut transversely at 15 microns. Ovaries were fixed in Craf solution, embedded, sectioned, mounted on 50 × 75 mm. slides, and stained with Delafield's haematoxylin, and a cover slip was applied.

The presence and behavior of the stigmatic membranes were observed with a stereoscopic microscope. Where it was entire, it could be teased to show that a pliable membrane covered the stigma and jagged pieces could be observed after it was ruptured.

Results

Data on number of ovules per ovary, number of seeds per pod, and self-fertility are presented in Table II. They show that all ovules regularly form seeds in some species and that one-half or less of the ovules form seeds in other

species. It is also evident that all species studied in which all ovules are fertilized are highly self-fertile, both with and without manipulation. The observation that all ovules in the self-fertile species *L. tetragonolobus* do not develop into viable seeds is not necessarily contradictory to the observed association between self-fertility and proportion of ovules fertilized as post-fertilization abortion may also reduce the number of viable seeds. Many ovules in the pods of *L. tetragonolobus* examined appeared to have started to develop but did not mature into viable seeds, which may indicate post-fertilization abortion. The species which are not self-fertile without manipulation vary in degree of self-fertility following manipulation. In these species about one-half or less of the ovules form seeds.

TABLE II

THE NUMBER OF OVULES PER OVARY AND OF SEEDS PER POD FOLLOWING CROSS-POLLINATION AND DEGREE OF SELF-FERTILITY WITHOUT AND WITH MECHANICAL SELF-POLLINATION IN *Lotus*

Species	Ovules per ovary	Seeds per pod	Self-fertility	
			Without manipulation	With manipulation
L. corniculatus	49.0	18.2	None	Slight
L. tenuis	42.0	21.0	None	Slight
L. uliginosus	48.8	26.4	None	Some
L. weilleri	48.8	21.6	None	High
L. suaveolens	32.4	15.7	None	High
L. jacobaeus	76.0	18.2	None	None
L. corniculatus var. *japonicus*	30.8	29.4	High	High
L. angustissimus	16.0	16.3	High	High
L. hispidus	13.3	13.0	High	High
L. palustris	11.5	10.0	High	High
L. filicaulis	22.0	23.0	High	High
L. parviflorus	26.8	21.4	High	High
L. divaricatus	19.2	15.8	High	High
L. perigrinus	19.0	19.3	High	High
L. tetragonolobus	51.3	8.7	High	High
Hosackia sp.	2.5	2.2	High	High

Giles (6) pointed out that manipulation of the flower of *L. corniculatus* serves to break a stigmatic membrane and to release fatty material onto the stigmatic surface. Pollen sticks to and germinates in this fatty material. He also reported that a stigmatic membrane is either fragile or not present in the autogamous species *L. ornithopodioides*, *L. divaricatus*, and one unidentified species. In the present study, a complete stigmatic membrane was observed on the stigmas of young flowers and buds of both self-sterile and autogamous species. In mature flowers, intact stigmatic membranes were found only with species which are not self-fertile without floral manipulation. These species appear to depend upon insect pollinators to break the stigmatic membrane. Ruptured stigmatic membranes were observed in undisturbed flowers of autogamous species. The stigmatic membrane appears to rupture automatically when flowers reach a certain stage of maturity so the self-pollen

can germinate and lead to self-fertilization. The time of self-pollination of autogamous species appears to be controlled by the time at which the stigmatic membrane ruptures.

Giles' (6) observations on the importance of the stigmatic fluid as a medium for germination and growth of pollen were confirmed. With *L. corniculatus*, in which adequate masses of pollen were applied, either self or cross, the stigmatic fluid dried up in 20 to 24 hours. Repeated pollination does not lead to fertilization once the stigmatic fluid is exhausted as is indicated by data presented in Table III. These illustrate that pollination and subsequent fertilization takes place during a relatively short time and that repeated pollinations do not lead to an increase in the proportion of ovules fertilized in each ovary.

TABLE III

SEEDS SET FOLLOWING SELF-POLLINATION, CROSS-POLLINATION, AND SELF-POLLINATION FOLLOWED IN 24 HOURS BY CROSS-POLLINATION ON A SELECTED CLONE OF *L. corniculatus*

Pollination	Total seeds	Total flowers pollinated
Self	2	34
Cross	43	8
Self followed in 24 hours by cross	0	34

If we exclude the *Hosackia* species and *L. tetragonolobus* (the only example examined of the subgenus *tetragonolobus*), we see in Table II that the number of seeds per pod ranges between 10 and 30 and the species which are not self-fertile without manipulation have evolved considerably higher numbers of ovules per ovary, which may serve to compensate for the reduction in the portion fertilized.

Data on ovule development in unpollinated ovaries are presented in Table IV. Developmental stages are illustrated in Figs. 1, 2, 5, and 6. The non-autogamous species *L. corniculatus*, *L. tenuis*, *L. suaveolens*, and *L. weilleri*

FIG. 1. A young ovule at the four nucleate stage. In ovules in which the nuclei were not observed, it was possible to classify them by observing the condition of other components.

FIG. 2. A mature ovule in one of the younger stages. Eight nuclei are present, the polar nuclei have not fused.

FIG. 3. A normal developing ovule in which the primary endosperm nucleus has undergone a number of divisions. This ovule was fixed 72 hours after pollination.

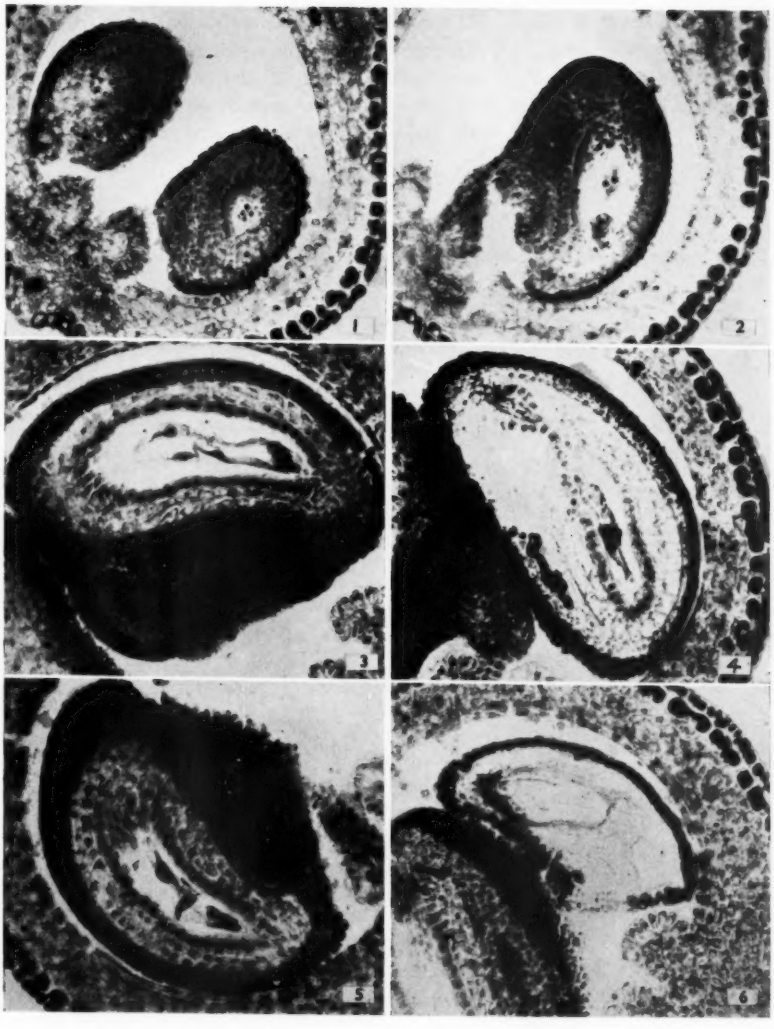
FIG. 4. A developing ovule in which the integumental tissue has developed rapidly and appears to be crowding the embryo sac. This ovule was fixed 72 hours after pollination.

FIG. 5. An old ovule in which the integumental tissue at the micropylar end is starting to disintegrate and a group of dark staining cells are present at the antipodal end of the cavity.

FIG. 6. A very old, presumably unfertilized, ovule typical of those found among developing ovules 72 hours after pollination.

NOTE: All microphotographs were taken at the same magnification and are reproduced at a magnification of approximately 200×.

PLATE I



have ovules in various stages of development within each ovary. The autogamous species *L. corniculatus* var. *japonicus*, *L. palustris*, and *L. divaricatus* have all ovules in the same stage of development within each ovary. As pollination takes place over a relatively short time span, it is apparent that all ovules of the non-autogamous species are not fertilized because they are not all in suitable developmental stages when pollen is available. Pollination occurs in autogamous species when the stigmatic membrane ruptures and this rupturing must occur when all ovules are fertilizable.

TABLE IV

OVULE DEVELOPMENT WITHIN INDIVIDUAL UNPOLLINATED OVARIES

	Species	Flower stage	Ovule development classes			Total ovules
			Young	Mature	Disintegrating	
1.	*L. corniculatus*	Bud	44	—	—	44
		"	34	—	—	34
		"	50	2	—	52
		"	25	23	—	48
		Young	14	23	1	38
		"	13	36	—	49
		"	3	37	4	44
		Medium	3	37	15	55
		"	—	33	18	51
		"	—	31	21	52
		"	—	39	23	62
		Old	—	27	22	49
		"	—	24	29	53
		"	—	4	51	55
		"	—	1	48	49
2.	*L. tenuis*	Medium	4	26	3	33
		"	1	28	3	32
		"	—	18	22	40
		"	—	18	23	41
		"	—	7	25	34
3.	*L. suaveolens*	Medium	4	28	1	33
		"	—	21	7	28
		"	—	20	8	28
4.	*L. weilleri*	Medium	—	23	10	33
		"	—	18	11	29
		"	—	16	12	28
5.	*L. corniculatus* var. *japonicus*	Medium	—	39	—	39
		"	—	36	—	36
		"	—	29	—	29
6.	*L. palustris*	Medium	—	14	—	14
		"	—	13	—	13
7.	*L. divaricatus*	Medium	—	16	—	16
		"	—	13	—	13

TABLE V

OVULE DEVELOPMENT WITHIN INDIVIDUAL POLLINATED OVARIES OF A PLANT OF *L. corniculatus* 72 HOURS AFTER POLLINATION WITH POLLEN FROM ITS PROGENY

Pollen sources	Mature ovules	Developing ovules	Integument in cavity	Old ovules	Very old ovules
The plant itself	19	1	4	25	5
	25	—	2	15	9
	36	—	1	11	3
Self-progeny 1	29	3	—	8	2
	40	2	—	2	6
	32	—	—	2	5
Self-progeny 2	26	2	1	4	15
	28	6	6	7	2
	35	3	1	8	—
Self-progeny 3	40	1	—	4	—
	25	—	—	11	—
	31	—	—	3	—
Self-progeny 4	23	11	—	6	4
	19	2	—	3	20
	27	6	2	3	5
Cross-progeny 1	34	—	—	10	—
	37	—	—	7	1
	38	—	—	4	—
Cross-progeny 2	24	—	—	34	2
	28	5	4	2	8
	29	—	—	3	5
Cross-progeny 3	26	—	—	2	16
	14	—	—	1	26

In the non-autogamous species, ovules in different stages of development are scattered at random throughout the ovaries. The progression in development of the ovules in each ovary of *L. corniculatus* as flowers mature is illustrated in Table IV. Although the visual classes may not correspond exactly to actual fertility classes, they should indicate the distribution of the fertility classes. The ovules classified in the young class (Fig. 1) had not undergone final nuclear division or the mature nuclear orientation had not been reached. Those in the disintegrating class (Figs. 5 and 6), showed visible evidence of disintegration. Ovules in both these classes are probably not fertilizable.

The time required for ovules of *L. corniculatus* to develop through the stages in these classes was estimated from the rate of change of corolla characteristics of some tagged flowers, which are compared to ovules collected from flowers in these stages and reported on in Table IV. Starting with the bud stage, which corresponds to Giles (6) pointed bud stage, 2 or 3 days elapsed before the young stage was reached, 4 to 7 days before the medium flower stage was reached, and 8 to 10 days before the flowers were considered old because the corollas wilted. Flowers in all these stages contained some ovules which appeared fertilizable. Therefore, ovaries of *L. corniculatus* contain some fertilizable ovules for 8 to 10 days, although individual ovules are probably

fertilizable for only 2 or 3 days. Variability in rate of ovule development of non-autogamous *Lotus* species extends the period during which each ovary contains some fertilizable ovules and compensates, at least partially, for capriciousness of pollinating insects in their visits.

Data collected from pollinated ovaries of *L. corniculatus* are in agreement with the hypothesis that random deviation in rate of ovule development is one reason why all ovules are not fertilized. These data, presented in Table V, indicate that this variability is not the only factor involved in low seed set following inbreeding. Observations in the present work agree with those of Giles (6), who found that self-pollen does not penetrate the ovary as readily as cross-pollen. In addition, a number of ovules were observed in which the integument appears to have developed more rapidly than is normal in relation to development of the embryo, leading to a condition (Fig. 4) similar to that described by Brink and Cooper (2) in *Medicago sativa*. Otherwise these data are in agreement with those presented in Table IV for unpollinated ovaries if allowance is made for development during a time lapse of 72 hours as these were collected 72 hours after pollination.

Discussion

In the previous section a relationship between variability in ovule development and self-sterility and the behavior of the stigmatic membrane is shown. The possible relationships with self-incompatibility will be considered in this section. According to Lewis (8), the main feature of an incompatibility system is that it causes a restriction on self-fertilization and certain cross-fertilizations. It does not necessarily lead to self-sterility. Bateman (1) described an incompatible species which apparently sets seed freely when flowers are manipulated to apply self-pollen. *L. weilleri* and *L. suaveolens* may behave in the same way. However, it is not possible to use the same technique as Bateman to determine if these species are self-incompatible as suitable genetic markers have not been found. If these species are self-incompatible, the division between self-incompatible and self-compatible species in *Lotus* may follow the division between those which are self-sterile and those which are self-fertile *without* floral manipulation. If this is so, the primary division between self-incompatibility and self-compatibility may be attributed to the behavior of the stigmatic membrane; if it breaks automatically at a certain stage of maturity we get self-compatibility, if it does not break until an insect visits the plant we get self-incompatibility.

From Bateman's (1) work, we see that a weak self-incompatibility system, that is, one which imposes only a slight restriction on self-pollen, may be an efficient outbreeding mechanism. In a self-incompatible *Lotus* species the stigmatic membrane may act as a starting gate to get self- and cross-pollen started at the same time and any slight advantage of cross-pollen may lead to a high degree of cross-fertilization. However, if the incompatibility medium is weak and insufficient cross-pollen is applied, there may be considerable seed set from self-pollination. Selective pressures should very soon evolve a desirable balance in each species or each population of each species.

From an evolutionary standpoint, the first step in the evolution of self-incompatibility in a *Lotus* species may be that the stigmatic membrane loses its ability to break automatically when the ovules mature. This makes the plants dependent upon insects to break the membrane and at the same time to apply cross-pollen. As cross-pollen develops the ability to reach the ovules ahead of self-pollen, we get a self-incompatibility system developing. At the same time the ovules may lose their uniformity in rate of development to compensate for variability in time of insect pollination and the number of ovules per ovary may increase to compensate for the resulting decrease in the proportion fertilized. This hypothesis may account for the observed differences in degree of self-sterility following self-pollination among the different species which the writer considers self-incompatible.

No reports of a relationship between a stigmatic membrane and self-incompatibility were found in the literature reviewed. However, reports in the literature of several studies on other Leguminosae suggest that variability in ovule development which may serve to prolong the period during which ovules may be fertilized may be quite common. Cram (4) reported *Caragana arborescens* has an average of 14.2 ovules per ovary and 3.4 seeds per pod following self-pollination. Dessureaux (5) found that *Trifolium repens* produces fewer seeds per pod than there are ovules per ovary and that selection for higher numbers of seeds per pod sometimes increased the number of ovules per ovary and sometimes increased the proportion fertilized. Cooper and Brink (3) found that an average of 66.2% of the ovules in *Medicago sativa* were fertilized following crossing. These examples indicate that a mechanism similar to the one described in *Lotus* may be quite common.

Acknowledgments

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METABOLISM OF THE INTERMEDIATE PRODUCTS OF STARCH AMYLOLYSIS IN DETACHED TOBACCO LEAVES[1]

S. Rizvi and G. Krotkov

Abstract

Detached tobacco leaves were placed on solutions of uniformly C^{14}-labelled glucose, "dextrins", and "oligosaccharides" which had been obtained by amylolysis of C^{14}-starch with ptyalin. All three carbohydrates were absorbed by the leaves, and their subsequent metabolism was found to be essentially the same. This observation eliminates the earlier suggestion that the absence of free maltose during the breakdown of starch in tobacco leaves is due to the fact that its precursors are exceptionally active and are metabolized away before they have a chance to give rise to free maltose.

Introduction

It was observed earlier (1) that there was no decrease in the specific activity of the C^{14}-maltose introduced into detached leaves when such leaves were placed in darkness and their starch was broken down. From this observation it was concluded that the breakdown of starch in tobacco leaves *in vivo* does not lead to the formation of free maltose.

Several tentative suggestions were made to explain these results. According to one, though the breakdown of starch *in vivo* is brought about by amylases, the earlier products are so active that they are metabolized as soon as they are formed and before they give rise to maltose. In order to test this suggestion it was decided to introduce some early products of starch amylolysis into tobacco leaves and to observe their metabolism.

Materials

Nicotiana tabacum, var. Connecticut seed leaf, plants were grown in pots on garden soil under a 16 hour day in a special constant light and temperature room.*

Uniformly labelled starch-C^{14} was hydrolyzed with saliva and the products were isolated chromatographically as described elsewhere (1). The products of starch amylolysis, which remained at the origin when the chromatogram was run in butanol:ethanol:water (52:32:16), were called "dextrins". The products, which run half way between the origin and maltose, were called "oligosaccharides". The only other products, which appeared after amylolysis, were maltose and glucose. The bands representing these compounds were eluted and made to volumes and they represented the fractions fed to tobacco leaves.

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* The authors wish to thank Dr. F. H. White, from the Tobacco Division, Central Experimental Farm in Ottawa, who kindly provided the seeds.

Experimental

Six uniform, mature tobacco leaves were detached. Two of them were placed separately on 1 ml. of glucose-C^{14} which was used as control, two on solutions of "dextrin", and two on "oligosaccharide". Leaves were left for 1 hour in strong electric light in front of a fan to stimulate absorption.

In order to observe whether there was any preferential uptake of the carbohydrates fed, as compared with water, six 10 lambda aliquots of each solution were plated and counted before and after feeding. The results are given in Table I. Apparently glucose, "oligosaccharide", and "dextrin" fractions all were taken into leaves at about the same rate as water. The amounts of the various substances fed, which were absorbed by the leaves during 1 hour of feeding, are shown in Table II. These were calculated by subtracting the

TABLE I

SPECIFIC ACTIVITIES OF THE SOLUTIONS BEFORE AND AFTER UPTAKE BY DETACHED TOBACCO LEAVES

Leaf No.	Carbohydrate offered	Initial activity	Final activity	Average final activity	Average percentage of variation
		Disintegrations/10 lambdas of the solution fed			
1	Glucose	127,732	128,968	132,755	+ 3.9
2	"	"	136,543		
3	"Oligosaccharide"	45,320	46,839	47,272	+ 4.3
4	"	"	47,705		
5	"Dextrin"	228,228	241,483	238,045	+ 4.3
6	"	"	234,607		

TABLE II

ABSORPTION OF VARIOUS CARBOHYDRATES BY DETACHED TOBACCO LEAVES*

Leaf No.	Carbohydrate offered	Amounts offered, mg.	Amounts absorbed, mg.	Amounts absorbed as % of those offered
1	Glucose	17.59	9.45	53.7
2	"	"	15.48	87.9
3	"Oligosaccharide"	1.00	0.54	54
4	"	"	0.83	83
5	"Dextrin"	5.03	1.62	32.2
6	"	"	1.70	33.9

* Amounts in the "dextrin" and "oligosaccharide" fractions are expressed as mg. of glucose. All carbohydrate analyses were made with anthrone using the technique of Morris (2).

amounts left in the solution after feeding from those offered initially. Substantial amounts of all carbohydrates offered were absorbed. Glucose and "oligosaccharide" were absorbed to about the same extent and the intake of "dextrin" was about one-half of these.

After 1 hour of feeding, one half of each leaf was cut off along the midrib and extracted with 80% ethanol. The other half, with the lamina still attached to the midrib, was transferred to water and kept for 6 hours either in light or in darkness. The half leaf kept in light carried on photosynthesis in air. The half leaf kept in darkness was aerated with CO_2-free air and its CO_2 production was determined.

At the end of 6 hours the leaves were extracted with 80% ethanol and analyzed for their carbohydrates. The technique of determining respiration, as well as various methods used in the separation and analysis of carbohydrates, were as those described earlier (1).

As is seen from Table III, the greatest amount of each carbohydrate appeared at the end of 6 hours in the alcohol insoluble fraction, less than 50% remained alcohol soluble, 2–4% was respired, and only a small amount was incorporated into starch.

TABLE III

METABOLISM OF VARIOUS CARBOHYDRATES BY DETACHED TOBACCO LEAVES

Leaf No.	Carbohydrate offered	Respired	In alcohol soluble	In starch	In the rest of the alcohol insoluble
		As % of the activity absorbed			
1*a*	Glucose	3.9	74.1	0.4	25.5
1*b*	"		41.1	0.1	54.9
2*a*	"		47.4	0.8	51.8
2*b*	"		48.2	0.8	51.0
3*a*	"Oligosaccharide"	3.8	57.5	0.1	42.4
3*b*	"		40.0	0.8	55.4
4*a*	"		57.3	0.3	42.4
4*b*	"		40.0	3.9	56.1
5*a*	"Dextrin"	2.2	57.4	0.9	41.7
5*b*	"		33.6	0.6	63.6
6*a*	"		33.2	0.1	66.7
6*b*	"		32.9	0.5	66.6

Discussion

From the data presented above, one can see no great difference between the three substances fed to leaves. All of them were absorbed to a considerable extent, their absorption rates were the same as water, and once taken into leaves their metabolic fate was essentially the same. None of them were effective starch formers. Analysis of the alcohol soluble fractions from the leaves indicated that their sugars were glucose, fructose, and sucrose, with the

last one usually predominating. There was no evidence that either "oligosaccharide" or "dextrin" fractions were unusually strong sucrose formers. Only between 2 and 4% of the activity fed appeared in the CO_2 respired, indicating that neither "oligosaccharide" nor the "dextrin" fraction were respired more preferentially than glucose.

It might be argued that though qualitatively all these carbohydrates were utilized essentially in the same way, the rates of "oligosaccharide" and "dextrin" utilization were much higher. Since the amounts of these two carbohydrates offered and absorbed by the leaves were considerably smaller than those of glucose, it is impossible to compare their metabolic rates directly.

Such a comparison, however, can be made in the following manner. By calculating the average absorption of each carbohydrate by all four leaf samples, one obtains the average amounts absorbed by 1 g. of leaf fresh weight per hour. In a similar manner one can calculate the average amounts of each carbohydrate which have appeared in the alcohol soluble and insoluble fractions. From these data one can calculate the amounts of "oligosaccharides" and "dextrins" which would appear in various metabolic fractions if the absorbed amounts of these two carbohydrates would be equal to the absorbed glucose. The results of such a calculation are given in Table IV.

From this table it is seen that the amounts of both "oligosaccharides" and "dextrins" respired or found in the alcohol soluble and insoluble fractions are essentially the same as those of glucose.

On the basis of all these data one has to conclude that the present experiments failed to reveal any evidence that either "oligosaccharide" or "dextrin" fractions, formed from the breakdown of starch by amylase, are more active

TABLE IV

A COMPARISON BETWEEN THE UTILIZATION RATES OF VARIOUS CARBOHYDRATES ABSORBED BY DETACHED TOBACCO LEAVES

Leaf No.	Carbohydrates offered	Average absorption per four samples, mg./g. leaf fr. wt.	Relative absorption	Respired	Alcohol soluble	Alcohol insoluble
				mg./g. leaf fresh weight/7 hr.		
1*a*	Glucose	2.56	100	90.5	1.336	1.201
1*b*	"					
2*a*	"					
2*b*	"					
3*a*	"Oligosaccharide"	0.14	5.5	90.9	1.218	1.218
3*b*	"					
4*a*	"					
4*b*	"					
5*a*	"Dextrin"	0.33	12.9	65.9	1.046	1.534
5*b*	"					
6*a*	"					
6*b*	"					

metabolically than glucose. In other words, our earlier failure to observe formation of free maltose under the conditions of starch breakdown in living tobacco leaves is not due to the higher metabolic activity of the earlier degradation products of starch.

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SOME EFFECTS OF INCREASING SOIL SALINITY ON PLANT COMMUNITIES[1]

LLOYD B. KEITH

Abstract

Analyses of halophytic vegetation and saline soils adjacent to three different-aged impoundments were conducted on a study area in southeastern Alberta. Two impoundments had been constructed by Ducks Unlimited (Canada), and were 4 and 8 years old. The third was a small natural lake. Mesic prairie vegetation and soils were also sampled to secure comparative data. The establishment of artificial impoundments has raised the water table, permitting a rapid upward movement of soluble salts into the surface soils from lower levels in the glacial drift. Four distinct halophytic communities were recognized: a *Hordeum* community around all three impoundments, a *Distichlis* community around the older two, and *Suaeda–Chenopodium* and *Salicornia* communities around the oldest (the natural lake). It is believed that as salt concentrations increase the mesic prairie community is replaced by a *Hordeum* community which in turn is replaced by a *Distichlis* community. Evidence of *Suaeda–Chenopodium* and *Salicornia* communities supplanting the *Distichlis* community is not clear cut and edaphic factors other than salinity per se are involved.

Introduction

Objective

The objective of this study was to investigate changes observed in terrestrial vegetation surrounding a number of artificial impoundments in southeastern Alberta. These vegetational changes were believed due to a progressive accumulation of soluble salts in the surface soil; hence, it was decided to study the relationship between soil salinity and plant communities. By comparing soil and vegetation around three impoundments of differing age, it was hoped in addition to secure information on the rate of soil and vegetational changes.

Since this particular study constitutes but one facet in a broad ecological investigation of waterfowl within the region, time would not permit an intensive exploration of all the factors associated with the above-mentioned phenomena. An attempt was made, however, to gather sufficient data to outline some of the major edaphic and floristic changes which have occurred.

Description of Study Area

Location

The study area lies on the prairies of southeastern Alberta, approximately 25 miles NNW. of the town of Brooks (Fig. 1). Its exact location is in townships 22 and 23, range 15, west of the 4th meridian.

Topography and Soils

The impoundments of the study area are situated in what appears to be an old shallow drainage way. During preglacial or early postglacial times this

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may have been part of the flood plain of the Red Deer River, 5 miles to the east. A topographic high of land running north and south and rising 40 to 50 feet above the plain demarks its western boundary. To the south and east partially stabilized sand dunes are visible. Northward the land fans out into gently undulating prairie.

The normal soil of the study area consists of alluvial sands which have been reworked by wind. There is little indication of profile development and the soil may be classed as an azonal fine to medium sand. In a natural depression, where soil moisture was considerably higher than elsewhere and drainage poor, a glei-type profile was found.

Glacial till from the Bearpaw Formation is intermittently exposed along the summit of the above-mentioned topographic high. Here the soil profile has a 4-in. A-horizon of brown fine sand; the B-horizon is about 2 in. thick, slightly darker, and of a medium to fine sand. Below this is a very prominent B_{Ca} of fine to loamy sand. Pebbles are scattered throughout the entire profile.

Bedrock below the glacial drift is Bearpaw Formation (15), a Cretaceous marine shale rich in soluble salts of calcium, magnesium, and sodium. While there is at present no information on the depth of the drift, a reasonable estimate based on drillings elsewhere would place it at between 15 and 40 ft.

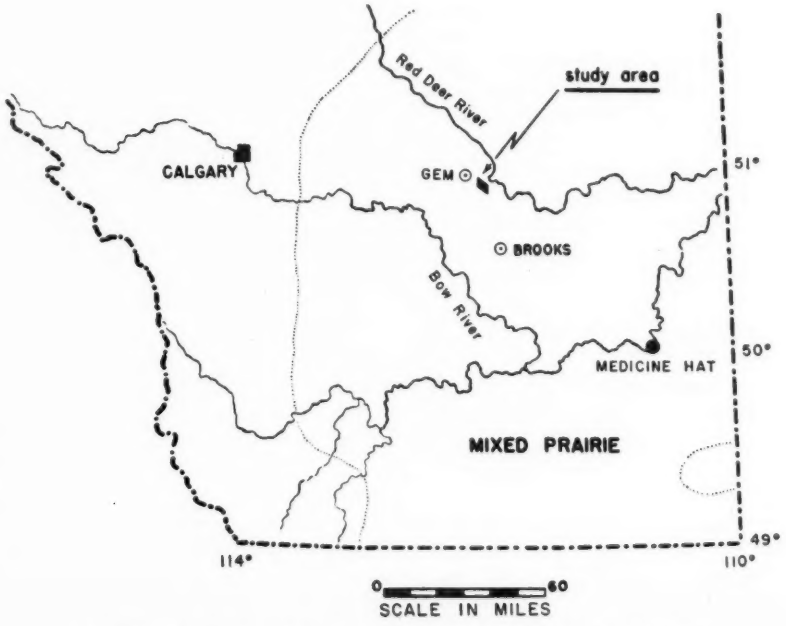


FIG. 1. Outline map of southern Alberta showing position of study area.

Climate

The climate of this region is characterized by hot, dry summers and cold winters with light snowfalls. The mean annual precipitation (20-year average) at Bassano, 22 miles SW. of the study area, was 13.57 in.* Of this, 9.72 in. fell as rain, and a moisture equivalent of 3.85 in. as snow. Almost 50% of the total yearly precipitation occurs during the months of May, June, and July. The annual daily mean temperature at Bassano was 40° F. Monthly means vary from 12° F. in January to 67° F. in July.

Marked differences in both temperature and precipitation may occur within and between years. For example, 35 miles to the ESE., at Jenner, the average yearly high temperature is 100° F., while the average yearly low is −42° F.* Precipitation at three stations (Jenner, Brooks, and Medicine Hat) near the study area averaged 21.89 in. during 1927, and in 1928 the mean for the same stations fell to 9.64 in. (15).

Desiccating chinook winds from the western mountains regularly sweep these plains, further aggravating the arid conditions and reducing spring run-off to a minimum. The frost-free period here is about 120 days.†

Natural Flora and Agriculture

Broadly speaking the natural flora of the region is Mixed Prairie with *Bouteloua gracilis* and *Stipa comata* as the principal dominants (8). On the study area, as discussed shortly, the soil's sandy texture and heavy grazing have modified the vegetation greatly.

Chief agricultural crops in the region are wheat, oats, barley, flax, field peas, and alfalfa. These are grown largely on irrigated farms. Irrigation water is brought through a series of canals from the Bow River, 30 miles SW. The only dry-land crops of significance are wheat, fall rye, and flax. Since the danger of drought here is great, dry-land farming is particularly hazardous.

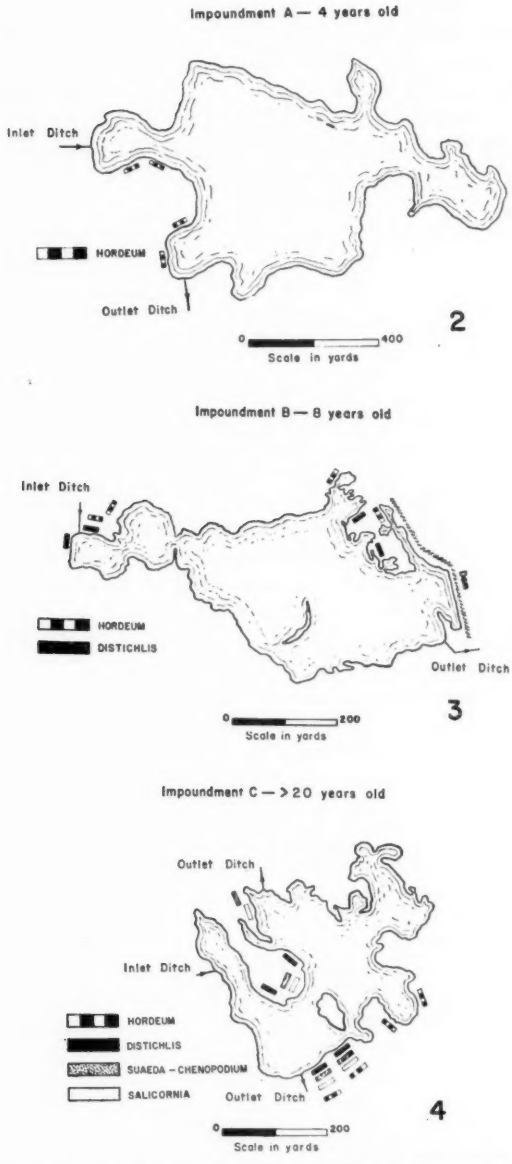
Extensive tracts of unbroken prairie are used as grazing land. Although there are some large private leases, most of this range is subdivided into community pastures. Under this arrangement, farmers in irrigated areas owning little pasturage are able to graze cattle at a set cost per head during the spring, summer, and early fall months.

History of Impoundments

The impoundments under study were constructed by Ducks Unlimited (Canada) between 1946 and 1952, on land used exclusively for grazing. These impoundments serve two purposes: first, they provide permanent waterfowl breeding habitat; second, they serve as additional stock-watering sites. Surplus water diverted from a local irrigation district spillway is utilized to maintain water levels. This is funneled through a number of artificial lakes and potholes and is eventually discharged into the Red Deer River drainage.

*Department of Transport, Meteorological Division, Toronto, Ontario. Climatic summaries for selected meteorological stations in the Dominion of Canada, Vol. I.

†Department of Transport, Meteorological Division, Toronto, Ontario. Climatic summaries for selected meteorological stations in Canada, Vol. III. 1956.



FIGS. 2, 3, 4. Impoundments on study area showing the approximate position of quadrats.

Paralleling the development of these permanent waters was an elevation in the local water table. Along the newly established shorelines and in low-lying areas it approaches the surface. The ramifications of this are important, because complete saturation of the sandy-textured soil has permitted a rapid upward movement of soluble salts. These have been further concentrated in the surface soil through evaporation.

The immediate source of these salts is not known for certain. It seems most likely, however, that they are situated at lower levels in the alluvial sand which forms the parent material for the area's soils. (Soil samples obtained from depths of 0 to 3 ft. on mesic sites yielded no evidence of soluble salts.) Accumulation of salts probably took place over a period of years through lateral movement from other localities, the initial source being either the Bearpaw Formation or glacial drift derived from it.

To determine whether spillwater used to maintain water levels constituted a major source of salts, analyses of samples of this water were conducted. The results were considered negative as no sample contained more than 195 p.p.m. soluble salts.

Observation, during the 3-year period 1953–55, of widespread vegetational changes believed to have been initiated by increasing soil salinity, prompted the present investigation which was conducted in July of 1956.

It should be mentioned at this point that not all impoundments, or all sections of every impoundment, exhibit signs of salinization in peripheral or adjacent soils. Numerous factors conceivably influence this process, including: depth and distribution of soluble salts, proximity of water table to the surface as governed by topography, rate and distribution of flow of ground waters, impervious layers, etc.

Methods

Plant communities around two artificial impoundments and a small natural lake were studied (Figs. 2, 3, 4). These areas were selected because preliminary inspection suggested that plant growth here was being affected by concentrations of free salts in the surface soils. One impoundment (A) was 4 years old, another (B) was 9 years old, and the natural lake (C) is known to have held water for more than 20 years. Their individual surface areas ranged from 30 to 60 ac. Data on water-level fluctuations are available for B-area only, and these only for the period since 1953. From 1953 to 1956 the average yearly variation was 20.3 in., while the greatest fluctuation in any one year occurred in 1954 and amounted to 30 in. It is believed unlikely that water levels in either of the other two areas have varied more than this, if indeed as much, over the same period. During the drought of the 1930's it is probable, however, that C-area was dry at least part of the year.

Species composition of mesic prairie similar to that which was present on sites A and B prior to flooding was also determined. Soil samples were collected in conjunction with vegetative data.

Vegetation was analyzed in the following manner:

1. The major halophytic communities around each body of water were first determined by visual inspection on the basis of general physiognomy and leading dominants.

2. Twenty 1-ft.-square quadrats were examined in each community, and the species found in each quadrat listed. Quadrats were taken in groups of five, on four different sites within a community. The scattering of quadrats in this manner provided a more representative picture of the community as a whole, and in addition facilitated sampling when the community was discontinuous, viz., not present in an uninterrupted belt. The position of each quadrat was determined as follows. Once within the community to be studied, the investigator oriented himself so that the nearest shoreline was on his left as he faced in a direction roughly paralleling it. From this position a stake was simply thrown forward and the lower left-hand corner of the first quadrat placed on the point touched by the stake's proximal end. Four subsequent quadrats were taken at 10-pace intervals along a straight line projected from this point and parallel to the shoreline. The above operation was repeated four times, each instance on a different site, for a total of 20 quadrats per community.

3. In determining species composition of the mesic prairie community, forty 1-ft.-square quadrats were taken. The sampling technique was similar to that described above for the halophytic vegetation, except that four groups of 10 quadrats were examined, instead of four groups of five. The position of the first quadrat was again determined by tossing a stake; the remaining nine quadrats were situated at 10-pace intervals, east of the first, and on an east–west line through it.

Soil samples were collected from within each plant community at depths of from 2 to 6 in. These samples were mixed together, air-dried, labelled, and set aside for later laboratory analysis at the University of Alberta Soils Department. Each of the soils was tested to determine pH, total soluble salts, and the relative amounts of various ions present. The standard procedures outlined in a United States Department of Agriculture publication (13) were employed.

Note was also taken of major changes in soil profiles on saline sites. However, much additional data should be gathered here before even a cursory description of the evolution of such profiles can be given.

Results and Conclusions

Table I presents the percentage occurrence of species found in 40 quadrats taken on the mesic prairie community. Two factors have contributed to the divergence of this vegetation from the characteristic *Bouteloua–Stipa* community as described by Moss (8). As previously indicated, the soil here is of a sandy texture; this encourages the growth of *Calamovilfa longifolia* (1), which becomes one of the leading dominants. Additional but less obvious changes in species composition are probably also effected by this edaphic factor.

Grazing too has played an important part in molding the present community. Within the past 30 years, this area has been subjected to severe

TABLE I

SPECIES COMPOSITION OF MESIC PRAIRIE

Species	% Occurrence (40 quadrats)
Calamovilfa longifolia	92.5
Bouteloua gracilis	82.5
Stipa comata	75.0
Agropyron smithii	35.0
Agrostis scabra	17.5
Koeleria cristata	5.0
Equisetum prealtum	15.0
Carex spp.*	100.0
Juncus balticus	7.5
Thermopsis rhombifolia	17.5
Vicia trifida	10.0
Glycyrrhiza lepidota	2.5
Opuntia sp.	2.5
Artemisia frigida	92.5
Chrysopsis villosa	17.5
Unidentified species	5.0

**Carex eleocharis, C. heliophila, C. obtusata, C. douglasii.*

grazing by horses and sheep. Only during the last 10 years has grazing pressure been controlled with a view to range improvement. Such palatable grasses as *Stipa comata* and *Koeleria cristata* have doubtless decreased in abundance, while *Calamovilfa longifolia* and the forbs *Artemisia frigida* and *Thermopsis rhombifolia* have increased (1). Coupland (2) indicates that heavy grazing and trampling favor increases in density of *Carex eleocharis*. Moss (9) reports that in the Peace River grasslands of Alberta, *C. heliophila* and *C. obtusata* became more abundant with grazing. Heavy grazing therefore has likely fostered the ubiquitous mats of *Carex* spp. present here.

A comparison of species found in the halophytic communities (3*a*, 11) around each of the impoundments is given in Table II. The number of salt-tolerant communities in an area increased with its age. A *Hordeum* community is found in all three areas, a *Distichlis* community occurs around impoundments B and C, and the *Suaeda–Chenopodium* and *Salicornia* communities are restricted to the oldest of the three (C-area).

From the pH and percentage soluble salts in soil samples collected within the above communities (Table III), it is patent that a marked increase in soil pH and salinity accompanied the establishment of permanent waters. According to Daubenmire (3*b*), soil pH may affect the availability of certain plant nutrients, while in some cases the hydroxyl ion concentration is sufficient to be toxic to plants (5). The principal effect of concentrations of soluble salts is that of physiological desiccation.

In the *Hordeum* community of A-area, few mesic prairie species remained after 4 years. A notable exception to this was *Agropyron smithii* which increased in abundance. This species is fairly tolerant of moderate levels of salinity. Of the original mesic prairie sedges, *Carex douglasii* persisted in greatest abundance.

TABLE II

COMPARISON OF SPECIES IN HALOPHYTIC PLANT COMMUNITIES AROUND AREAS A, B, AND C

Community	Species	A-area % occurrence (20 quadrats)	B-area % occurrence (20 quadrats)	C-area % occurrence (20 quadrats)
Hordeum	*Hordeum jubatum*	100	100	100
	Distichlis stricta	—	30	45
	Spartina gracilis	5	10	15
	Puccinellia nuttalliana	—	10	10
	Agropyron smithii	60	30	
	Agrostis scabra	10	—	
	Muhlenbergia asperifolia	15	—	
	Calamovilfa longifolia	—	5	
	Carex spp.*	75	35	
	Juncus balticus	10	—	
	Ranunculus cymbalaria	15	20	
	Aster ericoides	25	10	
	Aster sp.	—	10	
	Taraxacum officinale	65	30	
	Sonchus arvensis	30	40	
	Cirsium arvense	—	5	
	Solidago sp.	—	5	
	Misc. species	20	—	
Distichlis	*Distichlis stricta*		100	100
	Puccinellia nuttalliana		20	—
	Atriplex argentea			25
	Suaeda depressa			5
Suaeda–Chenopodium	*Suaeda depressa*			85
	Chenopodium salinum			75
	Salicornia rubra			50
	Distichlis stricta			35
Salicornia	*Salicornia rubra*			100

*Largely *Carex douglasii* in A-area.

TABLE III

TOTAL SALTS, pH, AND IONS IN SOILS UNDER PLANT COMMUNITIES

Area	pH	Ohms resist.*	% salts	Ca	Mg	Na	SO_4	Cl	Plant community
Prairie	5.7	912	neg.†	tr‡	2	—	—	—	Mesic prairie
A	7.6	122	0.26	25§	>6	++	450	<20	*Hordeum*
B	7.5	67	0.50	40	>6	+	600	50	*Hordeum*
C	6.8	64	0.52	20	>6	+‡	525	50	*Hordeum*
B	8.2	19	2.40	120	>6	++	>800	>200	*Distichlis*
C	8.2	36	1.00	200	>6	+‡	>800	130	*Distichlis*
C	7.1	26	1.45	tr	>6	+‡	>800	>200	*Suaeda–Chenopodium*
C	7.0	22	1.90	—	>6	‡‡	>800	>200	*Salicornia*

*Ohms resistance of saturated soil paste, an index of soil salinity (13).
†Negligible.
‡Trace.
§Parts per million.

Data in Table II suggest that pronounced changes in species composition have taken place within the *Hordeum* community with the passing of time. While increased salinity appears responsible for the presence of *Distichlis stricta* and *Puccinellia nuttalliana* in this community on B- and C-areas, the absence from C-area of a number of "weedy" species, i.e., *Taraxacum officinale*, *Sonchus arvensis*, *Aster ericoides*, etc., which occur on the *Hordeum* communities of A- and B-areas, is not explicable on the basis of changing soil salinity. Since the difference in soil salinity beneath the *Hordeum* communities of B- and C-areas is only very slight, it seems reasonable to assume that such "weedy" species have been eliminated from C-area by plant successions independent of further increases in salt concentrations per se.

The *Distichlis* community, present only in B- and C-areas, likely replaced *Hordeum* community species as salts accrued. As the process of salination progresses in A-area, a *Distichlis* community will undoubtedly arise here also. Salt concentrations in soils supporting B-area's *Distichlis* community averaged 2.40%. In C-area this community was situated on soils bearing 1.00% soluble salts.

The distribution of the four halophytic communities of C- area (Table III) is directly correlated with total soluble salts in the soil; however, soil moisture follows a similar gradient and water relationships, including aeration, may enter the picture as well (12, 14). The question now arises as to whether moister sections of the *Distichlis* community in B-area can be expected to develop through future plant successions into *Salicornia* and *Suaeda–Chenopodium* communities. Certain basic differences in these soils may mitigate this possibility. For example, the *Salicornia* and *Suaeda–Chenopodium* communities of C-area are situated on neutral soils (pH 7.0 and 7.1 respectively), whereas soils supporting the *Distichlis* community of both areas are considerably more alkaline (pH 8.2 in each case). This increased pH is coincident with a large increase in calcium in the latter soils (Table III). Calcium here occurs as $CaSO_4$, $CaCO_3$, and $Ca(HCO_3)_2$, the carbonate and bicarbonate forms being responsible for the increased pH.

It is not known why calcium is absent from the *Salicornia* and *Suaeda–Chenopodium* zones of C-area and yet so prevalent in soils of the immediately adjacent *Distichlis* community. It is possible that the shoreline of this natural impoundment has in the past advanced repeatedly to a point marked by the edge of the present *Distichlis* community, then subsequently retreated. In so doing, calcium may have been preferentially dissolved from the flooded area which is presently occupied by the *Suaeda–Chenopodium* and *Salicornia* communities. This is of course merely speculation; nevertheless, it would probably be unwise at this time to forecast any immediate successional displacement of *Distichlis* by *Salicornia* and/or *Suaeda–Chenopodium* communities on B-area's high calcium soils.

The limited quantity of data on profiles of saline soils collected during this study permits but a very superficial description of certain changes which have taken place. In general, there appears to be a pronounced darkening of the

upper stratum, the A_1-horizon being almost black in color and between 2 and 4 in. thick. A somewhat lighter A_2-horizon is also in evidence and extends to a depth of about 8 in. The B-horizon is again darker than the A_2, though not as dark as the A_1. It was found to vary from 4 to 8 in. in thickness. Below this a C-horizon, lighter in color than the B-horizon, is visible. Little textural difference was noted in these soils as compared with the azonal fine to medium sand from which they are assumed to have developed. Color changes indicated above seem to be caused principally by colloidal suspensions of organic matter.

The average salt content in the various horizons of two saline soil profiles in C-area was determined as follows: $A_1(>3.50\%)$, $A_2(2.02\%)$, $B(1.60\%)$, and $C(1.47\%)$. This clearly illustrates the tendency for salts to accumulate at the surface.

Discussion

One of the most striking features of soil salinization and coincident vegetational change on the study area is the rapidity with which it occurs once impoundments are constructed. Salt concentrations of 2.40% 8 years after flooding (B-area) clearly illustrate this. Furthermore, among the observations which initially led to this study was that even within a single year patches of shoreline vegetation were changing from a community dominated by *Juncus balticus*, growing on moist non-saline soils (6), to one predominately of *Hordeum jubatum*.

Previous workers (10, 4) have pointed out that *Distichlis*, *Salicornia*, and other halophytes are capable of growth over a wide range of soil salinity. Gates *et al.* (4) state: "Soil analyses showed some significant edaphic differences in soils occupied by various species. However, no species was restricted in distribution to a narrow tolerance for any specific soil factor." While data from the present study tend to corroborate the above statement, it is of interest that the *Distichlis* communities of B- and C-areas were each situated on soils with a pH of 8.2, even though salt concentrations here were 2.40% and 1.00% respectively. Comparison of pH and salinity of soils supporting the *Hordeum* communities of A- and B-areas, which are quite similar in species composition (Table II), again reveals that differences in pH are small compared to differences in total soluble salts present (Table III). This suggests that pH or some associated factor becomes increasingly important in modifying plant communities once a certain level of salinity has been attained.

As increased emphasis is placed on irrigation farming in western Canada, more attention is being given to soil salinization—commonly spoken of as "the alkali problem". Since much of the potential irrigable land is also latent "alkali", due to underlying marine shales and/or glacial drift derived from them, it is now mandatory that extensive soil surveys precede new irrigation developments. These surveys permit more accurate appraisals of proposed irrigation land, thus avoiding costly abandonments in later years.

There is a close parallel between this agricultural problem and the salinization of soils around artificial impoundments as here described. As indicated earlier, waterfowl production is the primary objective behind the development of these waters. At present it is not known whether vegetational changes elicited by accruing salinity in the vicinity of these impoundments are detrimental to waterfowl. Indications are, however, that, in Saskatchewan, usage of saline lakes by breeding waterfowl is far less than usage of comparable non-saline waters (7). Should this prove equally true for small impoundments, such organizations as Ducks Unlimited must then give it active consideration when planning and developing waterfowl breeding grounds and habitat restoration programs.

Acknowledgments

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A RAPID TEST FOR EXTRACELLULAR OXIDASE IN CULTURES OF WOOD-INHABITING HYMENOMYCETES[1]

MILDRED K. NOBLES[2]

Abstract

A rapid blueing, indicating the presence of an extracellular oxidase, occurs when an alcoholic solution of gum guaiac is applied directly to cultures of wood-inhabiting Hymenomycetes that are associated with white rots while no color change follows its application to cultures of species that cause brown rots. Parallel tests on 133 species with the gum guaiac solution and the standard Bavendamm method, in which cultures are grown on malt agar containing gallic or tannic acid, gave identical results for nearly 90% of the species. Hence the rapid alcoholic gum guaiac method is recommended for determining extracellular oxidase production where this characteristic is used as a basis for separation in the identification of fungus cultures.

Introduction

The production of an extracellular oxidase, identical with or similar to laccase, by cultures of those wood-decaying Hymenomycetes that cause white rots distinguishes them from the species that cause brown rots, the cultures of which, in general, do not produce this extracellular oxidase. By using a suitable test, therefore, cultures may be sorted into two groups, a separation which has been utilized in identifying such cultures since it was introduced by Campbell (3) in 1938. He based his method on results described by Bavendamm (1), who observed that brown diffusion zones, which he attributed to the action of an oxidase, were formed when cultures of certain species known to cause white rots were grown on media containing gallic or tannic acid but were not produced by other species that are associated with brown rots. Using the same media, Davidson, Campbell, and Blaisdell (5) tested 210 species and found a similar correlation, 80% of the species that produced no diffusion zones being associated with brown rots, 96% of those that produced diffusion zones being associated with white rots. Hence, by using differential media containing gallic or tannic acid, cultures can be sorted into two groups, those that produce diffusion zones and those that do not. This basis for separation has been widely used in the identification of cultures by Davidson and Campbell (4), Davidson, Campbell, and Vaughn (6), Davidson and Lombard (7), Davidson, Lombard, and Hirt (8), Harmsen (11), Nobles (18, 19, 21), and others, following its original use by Campbell (3).

To avoid the contaminations likely to occur with the Bavendamm method where the gallic or tannic acid must be added to the malt agar following sterilization and where the media must be used in Petri dishes, Preston and McLennan (24) used differential media containing the dyes gentian violet or neutral red in culture tubes. A separation of cultures comparable to that

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based on the Bavendamm test was obtained, since the fungi that cause white rots decolorized the dyes and those that caused brown rots produced no color change. Preliminary experiments indicated that the color changes induced by the white rot fungi resulted from the oxidation of the dyes by an extracellular enzyme system. Law (15) tested extracts of the mycelia of 11 of the fungi used by Preston and McLennan on various substrates and found evidence of the presence in the species associated with white rots of a specific phenol oxidase similar in certain respects to laccase. Meanwhile Fahraeus (10) had cultivated a number of wood-rotting fungi on agars containing various phenols and amines and had found "that the range of substrates oxidized by the white rot fungi agrees well with that of the enzyme 'laccase' " but, because he had not studied the purified enzyme, he could not present definite conclusions. Later Lindeberg and Fahraeus (16) examined the extracts of mycelia of *Polyporus zonatus* and *P. versicolor*, which cause white rots, and the nutrient solutions on which they had grown and concluded that the "phenol oxidases formed by the mycelia of *Polyporus zonatus* and *P. versicolor* are of the laccase type. In both species the enzyme is mainly extracellular in occurrence."

Boidin (2) tested a number of substrates oxidizable by laccase in a search for an indicator that would resist sterilization, that would give a readily recognizable color change in the presence of laccase, and that would give consistent results comparable to those obtained with the Bavendamm test. Guaiacol in malt agar at pH 4.5 fulfilled these requirements and Boidin proposed that this guaiacol medium should replace the gallic acid medium in general use. He made comparative tests on guaiacol medium and on Bavendamm's gallic acid medium with 133 species of Hymenomycetes and in most cases found satisfactory agreement in the results. Boidin did not record the type of decay associated with the fungi that he tested but, since previous investigators have shown that a positive reaction on gallic acid medium is characteristic of those species that cause white rots and since positive reactions on gallic acid and guaiacol media indicate the presence of laccase, it is reasonable to assume that laccase is produced by the fungi that cause white rots. Boidin has noted that biochemists recognize some differences between the purified enzyme from the lac tree and that from fungi but that, for the present, he has continued to use the term laccase to designate the enzyme responsible for the oxidation of guaiacol, of hydroquinone, etc., by fungi.

An additional method for differentiating between species of Hymenomycetes that cause brown rots and those that cause white rots was recommended by Jorgensen and Vejlby (14). To overcome the inconsistent or questionable reactions obtained for certain species with the Bavendamm method, they used red cabbage extract, incorporated in the medium on which the cultures were grown or added to well-grown cultures in tubes. In either method the original purple color was changed to yellow by those species that produce extracellular oxidase and to red by the species that produce no such oxidase. They reported precise and consistent results for the 23 species that they tested. Recently Etheridge (9) reported that a casein hydrolyzate medium containing

wood meal from which the phenolic substances had been removed showed a browning reaction when inoculated with cultures of fungi that cause white rots but no such color change with species that are associated with brown rots. In comparative tests with isolates of 10 species, he obtained more reliable results with this medium than with the Bavendamm media.

In summary, then, various differential media can be used to separate the cultures of the Hymenomycetes that cause white rots from those that cause brown rots. The color changes induced by the former are attributed to the action of an extracellular phenol oxidase, which has been identified as laccase. It should be noted that Law (15), Lindeberg and Holm (17), and others have recognized tyrosinase also in extracts of fungus cultures of species associated with both white and brown rots but have described it as intracellular and so without effect on indicators in culture media. Higuchi and Kitamura (13), on the other hand, stated that "extracellular tyrosinase was recognized for some species. Then, there are some probabilities that Bavendamm's reaction is caused by both laccase and tyrosinase for these species at least. However, as a rule, the content of tyrosinase and degree of secretion are little compared with laccase."

Results

In the identification of many cultures of wood-decaying Hymenomycetes at the Ottawa Laboratory of the Botany and Plant Pathology Division, Bavendamm's method has proved satisfactory and valuable. Most species give consistent reactions and in those in which inhibition of growth occurs, this provides additional information of diagnostic importance. However, the preparation of the media is laborious and the inoculation and incubation of cultures is time-consuming. Jorgensen and Vejlby's more rapid method was tried but proved unsatisfactory, probably because of the difficulty of preparing a standard red cabbage extract. Consequently a search was made for a rapid test for laccase.

Since gum guaiac is a standard indicator for laccase-type oxidase, freshly prepared aqueous and alcoholic solutions of gum guaiac were added to water extracts of cultures, including mycelium and agar, of species known to give a positive test for oxidase with other indicators and, as a check, to potato slices. In all, the characteristic blue color that indicates the presence of oxidase was produced. Since the alcoholic solution gave satisfactory results and since it is stable for long periods while aqueous solutions deteriorate rapidly, only alcoholic solutions were used in subsequent tests. The concentration of 0.5 g. gum guaiac (obtained from the Fisher Scientific Company) in 30 cc. of 95% alcohol, recommended by Hawk (12), was used. This produces a pale brownish solution from which the non-soluble residue was removed by filtering. This preparation in dropper bottles has retained its potency for 6 months or longer.

In preliminary tests the alcoholic gum guaiac solution was added to aqueous extracts of cultures and was dropped on the surface of actively growing cultures on malt agar in Petri dishes or culture tubes. When the solution

was applied to cultures of species that give a positive test for laccase with gallic or tannic acid agars, a blue color appeared, usually within 2 or 3 minutes, whereas its addition to species that give a negative test with gallic and tannic acid agars produced no color change. Since the direct application of the gum guaiac solution gave satisfactory results, this method was adopted.

To compare results obtained with an alcoholic solution of gum guaiac with those from the Bavendamm test, both methods were applied to cultures of 133 species, made up of 83 species of Polyporaceae, 30 of Thelephoraceae, seven of Hydnaceae, and 13 of Agaricaceae. For most species, three isolates were used. Each isolate was grown on Difco malt agar in Petri dishes for 2 weeks, the inocula being placed at the center of the plates. From the actively growing mycelium on one of the plates, inocula were transferred to gallic and tannic acid agars in Petri dishes and incubated for 1 week. At that time the results of the Bavendamm test were recorded in the usual manner and gum guaiac solution was dropped on three areas between the margin and the inoculum of the 3-weeks-old cultures on malt agar. At the same time two or three drops of the gum guaiac solution were added to the stock cultures, of varying ages, from which the original transfers to malt agar had been made. The reactions were assessed as negative (−) or positive and rated as (+) to (+++++) depending on the intensity of the blue color developed, an attempt being made to relate the rating to that given for the Bavendamm test.

Of the 133 species, 85 gave positive reactions with both the Bavendamm test and the gum guaiac solution method, 29 gave negative results with both tests, and 19 gave inconsistent or questionable results with one or both methods. These results are summarized in Tables I to III.

Fifty-eight of the species in Table I, followed by reference numbers, had been studied previously by other authors and had been reported to produce diffusion zones with gallic and tannic acid agars by Davidson, Campbell, and Blaisdell (5) or give positive tests for laccase by Boidin (2), Fahraeus (10), Higuchi and Kitamura (13), or Lindeberg and Fahraeus (16). It appears, therefore, that the direct application of alcoholic gum guaiac to an actively growing culture gives results comparable with those obtained by other methods. Furthermore, of the species that gave this positive test for laccase, 56 are known to be associated with white rots, 49 from records supplied by Davidson, Campbell, and Blaisdell (5) (the type of rot caused by *Phlebia merismoides* was not known to them), five by Overholts (22, 23), and two by Nobles (20, 21).

Eleven of the species in Table II, followed by the reference number (5), were listed by Davidson *et al.* as being associated with brown rots and giving a negative test on gallic and tannic acid agars, while *Polyporus dichrous* was described as causing a white rot but giving a negative reaction. Regarding this species Overholts (23) wrote: "The decay usually seen associated with it is white, but careful dissection often shows a small amount of definitely brown rot in the vicinity of the sporophores. The inference is that the fungus decays extremely slowly and usually becomes surrounded by the decay

TABLE I

SPECIES SHOWING POSITIVE REACTIONS WITH THE BAVENDAMM TEST AND THE GUM GUAIAC TEST

Polyporaceae

Daedalea confragosa Bolt. ex Fr. (5)
D. unicolor Bull. ex Fr. (5, 13)
Fomes annosus (Fr.) Cooke (5, 13)
F. conchatus (Pers. ex Fr.) Gill. (2, 5)
F. connatus (Weinm.) Gill. (5)
F. everhartii (Ell. & Gall.) v. Schrenk & Spaulding
F. fomentarius (L. ex Fr.) Kickx (2, 5, 13)
F. igniarius (L. ex Fr.) Kickx (2, 5, 13)
F. igniarius var. *populinus* (Neumann) Campbell (5)
F. pini (Thore ex Fr.) Karst. (5)
Ganoderma applanatum (Pers. ex Wallr.) Pat. (5, 13)
G. oregonense Murr. (5)
Lenzites betulina (L. ex Fr.) Fr. (5, 13)
Phlebia mellea Overh.
P. merismoides Fr. (5)
P. strigosozonata (Schw.) Lloyd (5)
Polyporus abietinus Dicks. ex Fr. (2, 5)
P. albellus Peck (5)
P. amorphus Fr.
P. anceps Peck (5)
P. borealis Fr. (5)
P. cinnabarinus Jacq. ex Fr. (5)
P. conchifer (Schw.) Fr. (5)
P. croceus Pers. ex Fr. (5)
P. cuticularis Bull. ex Fr. (2, 5)
P. dryophilus var. *vulpinus* (Fr.) Overh. (2, 5)
Polyporus frondosus Dicks. ex Fr. (5, 13)
P. glomeratus Peck (5)
P. hirsutus Wulf. ex Fr. (2, 5, 13)
P. montanus (Quél.) Ferry
P. pargamenus Fr. (5, 13)
P. pubescens Schum. ex Fr. (5)
P. resinosus Schrad. ex Fr. (5)
P. sanguineus L. ex Fr. (13)
P. tomentosus Fr. (2, 5)
P. tomentosus var. *circinatus* (Fr.) Sartory & Maire (22)
P. versicolor L. ex Fr. (5, 9, 13)
P. volvatus Peck (5)
P. zonatus Fr. (16)
Poria albipellucida Baxter
P. decolorans (Schw.) Cooke
P. ferrea (Pers.) Bourd. & Galz. (2, 22)
P. ferruginea-fusca Karst. (22)
P. laevigata (Fr.) Karst. (2, 5)
P. lenta Overh. & Lowe
P. nigrescens Bres. (5)
P. notata Overh. (22)
P. obliqua (Pers.) Karst (2, 5)
P. odora (Peck) Sacc.
P. subacida (Peck) Sacc. (5)
P. tsugina (Murr.) Sacc. & Trott. (22)
P. weirii (Murr.) Sacc. & Trott. (5)

Thelephoraceae

Corticium furfuraceum Bres.
C. galactinum (Fr.) Burt (5)
C. polygonium Pers.
Gloeocystidium karstenii Bourd. & Galz.
Peniophora aspera (Pers.) Sacc.
P. cinerea (Pers. ex Fr.) Cooke (2, 5)
P. mutata (Peck) Höhn. & Litsch.
P. pithya (Pers.) J. Eriksson (2)
P. separans Burt
Peniophora septentrionalis Laurila (21)
Stereum chailletii Pers. (2)
S. hirsutum Willd. ex Fr. (2)
S. murraii (Berk. & Curt.) Burt (2, 5)
S. ochraceoflavum (Schw.) Ell.
S. pini (Schleich. ex Fr.) Fr. (2, 5)
S. purpureum (Pers. ex Fr.) Fr. (2, 5)
S. rufum (Fr.) Fr.
S. sanguinolentum (Alb. & Schw. ex Fr.) Fr. (2)
S. sulcatum Burt
Vararia granulosa (Pers. ex Fr.) Laurila

Hydnaceae

Echinodontium tinctorium Ell. & Ev. (2, 5)
Hericium erinaceus (Fr.) Pers. (5)
Odontia bicolor (Alb. & Schw. ex Fr.) Bres. (2, 20)
Radulum casearium (Morgan) Lloyd
Steccherinum septentrionale (Fr.) Banker

Agaricaceae

Armillaria mellea Vahl ex Fr. (2, 5, 13)
Flammula conissans Fr.
Omphalia campanella Fr.
Pholiota adiposa Fr. (5)
Pholiota spectabilis Fr.
Pleurotus ostreatus Jacq. ex Fr. (5)
P. serotinus Fr. (2, 5)
P. subareolatus Peck

TABLE II

SPECIES SHOWING NEGATIVE REACTIONS WITH THE BAVENDAMM TEST AND THE GUM GUAIAC TEST

Polyporaceae

Fomes officinalis (Vill. ex Fr.) Faull (5, 13)
F. pinicola (Sw. ex Fr.) Cooke (2, 5)
Lenzites saepiaria (Wulf. ex Fr.) Fr. (2, 5)
Merulius himantioides Fr.
M. lacrymans Jacq. ex Fr. (7, 13)
Polyporus albidus Schaeff. ex Secret. (13)
P. betulinus Bull. ex Fr. (2, 5)
P. dichrous Fr. (5)
P. sulphureus Bull. ex Fr. (2, 5)
Poria asiatica (Pil.) Overh. (22)
Poria carbonica Overh.
P. cocos Wolf (5)
P. monticola Murr. (18)
P. vaillantii Fr. (5)
P. xantha Fr. (22)
Trametes americana Overh. (5)
T. heteromorpha (Fr.) Bres. (5)
T. sepium Berk. (23)
T. serialis Fr. (5)
T. variiformis Peck (23)

Thelephoraceae

Coniophora puteana (Schum. ex Fr.) Karst. (2, 5)
Corticium fuscostratum Burt (25)
C. laeve Pers. (2)
C. vellereum Ell. & Crag.
Peniophora luna Rom.
P. resinosa Jackson
Stereum abietinum (Pers. ex Fr.) Fr. (2)
Trechispora brinkmannii (Bres.) Rog. & Jackson (2)

Agaricaceae

Lentinus lepideus Fr. (5)

TABLE III

SPECIES SHOWING INCONSISTENT REACTIONS WITH THE BAVENDAMM TEST AND THE GUM GUAIAC TEST

Fungus	Type of rot	On gallic acid medium: Reaction	On gallic acid medium: Growth, mm.	On tannic acid medium: Reaction	On tannic acid medium: Growth, mm.	Gum guaiac
Polyporus adustus Willd. ex Fr. (2, 5)	White	− to ++	20–40	− to +	15–25	++++
P. fumosus Pers. ex Fr. (5)	White	− to +	15–32	− to +	Tr–15	++ to ++++
P. tulipiferae (Schw.) Overh. (5)	White	− to ++	40–60	− to +	Tr–30	++ to ++++
Peniophora gigantea (Fr.) Massee (2, 5)	—	+ to +++	12–19	−	0	++ to ++++
Hericium abietis	—	+ to ++	0	−	0	+++
H. laciniatum Leers ex Banker	—	+++	0	−	0	+ to +++
Collybia velutipes Fr. (2, 5)	White	− to +	0	−	0	− to ++
Coprinus micaceus Fr.	—	− to ++	0	− to +	0	+ to +++
Schizophyllum commune Fr. (2, 5, 13)	White	−	Tr–37	+++	Tr–32	+++
Fomes roseus (Alb. & Schw. ex Fr.) Cooke (5)	Brown	− to +	15–25	− to +	Tr–15	−
F. subroseus (Weir) Overh. (2, 5)	Brown	− to +	15–30	−	10–25	− to +
Polyporus balsameus Peck (5)	Brown	− to +	15–25	−	Tr	+ to +++
Poria corticola (Fr.) Cooke	—	+++	45–70	−	Tr	− to ++
Peniophora phlebioides Jackson & Dearden	—	+++	45–55	−	0	− to ++
Claudopus nidulans (Fr.) Karst.	—	−	0	− to ++	0–Tr	− to ++
Merulius ambiguus Berk.	—	−	24–30	−	0	++ to +++
Polyporus guttulatus (Peck) (5)	Brown	−	15–35	−	0	++ to ++++
P. schweinitzii Fr. (5)	Brown	−	15–35	−	0	++++
Trechispora raduloides (Karst.) Bourd. & Galz.	—	−	20–30	−	0	+++

produced by other species of fungi." Thus the negative test for laccase is probably a true indication of the type of decay caused by this species. In addition to the 12 species listed by Davidson *et al.* eight, followed by the appropriate reference numbers, have been reported as causing brown rots by Davidson and Lombard (7), Nobles (18), Overholts (22, 23), and White (25). No laccase was found in 11 of the species in Table II that were tested by Boidin (2) or Higuchi and Kitamura (13). Hence absence of blueing when alcoholic gum guaiac is added to a fungus culture shows lack of laccase and also indicates that the fungus is one which causes a brown rot.

Of the 19 species listed in Table III, 15 gave inconsistent, negative to positive reactions with the Bavendamm test and of these, five also gave inconsistent results with the gum guaiac solution method, one gave a negative reaction, and nine gave a positive reaction. The remaining four species showed a negative test with the Bavendamm method but a positive test with the alcoholic gum guaiac method. Davidson, Campbell, and Blaisdell (5) tested 11 of the species and recorded inconsistent results for all except *Polyporus guttulatus* and *P. schweinitzii* while Boidin (2) tested five of the species and obtained inconsistent results with all but *P. adustus*, which gave him a negative test for laccase. These results suggest that in these species the production of extracellular oxidase is erratic or limited.

Five species, *Polyporus adustus*, *P. fumosus*, *P. tulipiferae*, *Collybia velutipes*, and *Schizophyllum commune*, are associated with white rots but give reactions with gallic and tannic acid agars which Davidson, Campbell, and Blaisdell (5) recorded as (−) to (++) with the note that "These fungi may require from 7 to 14 days to form brown diffusion zones, but the reactions are always positive." Four species, *Peniophora gigantea*, *Hericium abietis*, *H. laciniatum*, and *Coprinus micaceus*, for which the type of rot is not known, gave positive reactions on gallic acid agar, negative on tannic acid agar. For all nine species rapid blueing followed the application of alcoholic gum guaiac to the culture so that, for these species, this method is a more reliable test for extracellular oxidase than the Bavendamm method.

Fomes roseus, *F. subroseus*, and *Polyporus balsameus* are associated with brown rots but are erratic in their behavior on gallic and tannic acid agars. Davidson *et al.* wrote concerning them: "Reaction usually negative. However, a faint brown diffusion zone may form under center of mat. The same isolation may be negative at one trial but slightly positive at another." The slight browning on these media containing gallic and tannic acids may be the same as that produced on malt agar by these species and may be no indication of oxidase formation. No color change occurred when gum guaiac solution was added to cultures of *Fomes roseus*, a further indication that extracellular oxidase is not formed by this species, but weak to moderately strong blueing followed its addition to *F. subroseus* and *Polyporus balsameus* cultures. *Peniophora phlebioides* and *Poria corticola* produce moderately strong diffusion zones on gallic acid agar but none on tannic acid agar, and *Claudopus nidulans* produces no diffusion zones on gallic acid agar and no diffusion zones or weak

ones on tannic acid agar. When alcoholic gum guaiac was added to the cultures, some isolates of each showed no color change, others showed faint blueing after 30 minutes or longer in some areas. For these six species equally inconsistent results were obtained with the Bavendamm method and the gum guaiac method.

Finally, four species, *Polyporus guttulatus* and *P. schweinitzii*, which cause brown rots, and *Merulius ambiguus* and *Trechispora raduloides*, for which the type of rot is not known, gave negative tests with gallic and tannic acid agars but rapid and strong blueing with gum guaiac solution. In these species it seems unlikely that an extracellular oxidase of the laccase type is present since consistently negative results are obtained with gallic and tannic acid media. Higuchi and Kitamura (13) reported extracellular tyrosinase in cultures of *Polyporus schweinitzii* which suggests that this oxidase may be responsible for the color change in this species and perhaps in the other species of the group.

In summary, then, in parallel tests for extracellular oxidase with alcoholic gum guaiac and gallic and tannic acid agars, 113 or nearly 85% of the species tested gave identical reactions, either positive or negative; nine gave definite positive results with gum guaiac and inconsistent reactions, usually positive after some delay, with gallic and tannic acid agars; one gave negative results with gum guaiac and inconsistent results with gallic and tannic acid agars; five gave equally inconsistent results with both methods; and four gave contradictory results with the two methods. These results show that the direct application of alcoholic gum guaiac to actively growing cultures gives a reliable test for extracellular oxidase. In the standard procedure used in identifying cultures of wood-rotting Hymenomycetes it has been used in place of the Bavendamm method for several months and has proved satisfactory. It is therefore recommended for determining extracellular oxidase production where this characteristic is used as a basis for separation in the identification of fungus cultures.

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NATURAL AND ARTIFICIAL ×ELYMORDEUM HYBRIDS[1]

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Abstract

× *Elymordeum* Lepage is a validly published name for hybrids of *Elymus* L. × *Hordeum* L.; × *Hordelymus* should not be used as a name for these hybrids. Eight × *Elymordeum* hybrids are listed in this paper: × *Elymordeum dutillyanum* Lepage (*Elymus mollis* subsp. *mollis* × *Hordeum jubatum*); × *Elymordeum* **schaackianum** hybr. nov. (*Elymus hirsutus* × *Hordeum brachyantherum*); × *Elymordeum* **piperi** hybr. nov. (*Hordeum jubatum* × *Elymus triticoides*); × *Elymordeum* **dakotense** hybr. nov. (*Hordeum jubatum* × *Elymus canadensis*); !× *Elymordeum* **triploideum** hybr. nov. ($2n=21$) (*Hordeum distichon* × *Elymus racemosus* var. *racemosus*); !× *Elymordeum* **stebbinsianum** hybr. nov. (*Hordeum brachyantherum* × *Elymus glaucus*); !× *Elymordeum* **berkeleyanum** hybr. nov. (*Hordeum brachyantherum* × *Elymus condensatus*); and !× *Elymordeum* **montanense** (Scribn. in Beal) comb. nov. (*Elymus virginicus* × *Hordeum jubatum*). Most specimens of !× *Elymordeum montanense* are nm. *montanense*; two specimens with pubescent lemma backs are nm. **pubescens** nm. nov. The parentages of the four hybrids designated by !× have been experimentally confirmed.

Most of the parental species of *Elymus* and *Hordeum* were tetraploid ($2n=28$); the exception was the diploid, ($2n=14$), *Hordeum distichon*. One artificial hybrid, !× *E. triploideum*, was from Russia; the other hybrids were natural or artificial hybrids from North America. Seven of the parental species of *Elymus* and *Hordeum* are native in North America; two occur in Northeastern Asia and North America, one is native in Central Asia and one parent is a cultivated barley. In natural and artificial F_1 × *Elymordeum* hybrids, the anthers did not dehisce, the pollen was completely bad, and the spikes never set seed.

The natural hybrids of !× *Elymordeum montanense* occur rarely and sporadically from Montana to Nova Scotia. An artificial F_1 hybrid was produced in 1954–55 from the cross, *Elymus virginicus* L. f. *virginicus* × *Hordeum jubatum* L. var. *jubatum*. The artificial hybrid was identical with the natural hybrid from Lower Onslow, Nova Scotia. The hybrids and parental plants were tetraploid, $2n=28$, and there were many meiotic irregularities in the pollen mother cells.

Introduction

The purpose of this paper is to give a taxonomic treatment of known hybrids of *Elymus* L. × *Hordeum* L. and to record cytogenetic data for the artificial and natural hybrids of *Elymus virginicus* × *Hordeum jubatum*. Lepage (19) has recently validly published the name × *Elymordeum* for these intergeneric hybrids.

My interest in × *Elymordeum* hybrids began in 1952 when Dr. W. G. Dore of this Division drew my attention to the occurrence of *Hordeum montanense* Scribn. in Beal at Lower Onslow, Nova Scotia. Dore and Roland (9) recorded this station and Covas (8) suggested that the Lower Onslow specimens were "presumably intergeneric hybrids" and were "probably *H. jubatum* L. × *Elymus sp.*" A preliminary study indicated to me that the specimens of *Roland 41076* from Lower Onslow were probably hybrids of *Elymus virginicus* L. × *Hordeum jubatum* L. Collections of living material were made at Lower Onslow by D. Erskine and A. E. Roland in August, 1952. The plants of *E. virginicus* (Fig. 1*a*, 1*b*), *H. jubatum* (Fig. 2), and the presumed natural hybrid (Fig. 4) were grown in the greenhouse and plots of the Botany and

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Plant Pathology Division at Ottawa. In 1954–55, a single artificial F_1 hybrid seedling was grown from the cross, *E. virginicus* × *H. jubatum*. The artificial hybrid (Fig. 3) was identical with the natural hybrid (Fig. 4) from Lower Onslow, Nova Scotia. Covas (8) had already suggested that *Hordeum montanense* was "quite probably a sterile intergeneric hybrid involving *Hordeum jubatum* L. and *Elymus virginicus* L."

During the writing of the present paper, references were noted in the literature that mentioned five other ×*Elymordeum* hybrids and the author was able to examine on loan most of the herbarium specimens listed by these authors. In addition, Dr. George B. Van Schaack kindly sent specimens of a new ×*Elymordeum* hybrid and a specimen of another new artificial ×*Elymordeum* hybrid produced by Dr. G. L. Stebbins, Jr., was among the specimens loaned from the University of California. All these hybrids are included in the present treatment of ×*Elymordeum*.

Taxonomy of ×*Elymordeum*

×ELYMORDEUM Lepage, Nat. Can. 84:97–102. 1957.

×*Elyhordeum* Mansf. ex Stubbe apud Zizin et Petrowa in Der Züchter. 25:164. 1955. Nomen invalidum (no Latin description) based on × *Hordelymus* sensu Bacht. et Dar., nomen invalidum (no Latin description), name for an artificial hybrid, *Hordeum* L. × *Elymus* L., in Botan. Zhurn. Akad. Nauk SSSR 35:188–191. 1950. nec *Hordelymus* Jessen ex Harz in Samenkunde 2:1147. 1885.

Formula: *Elymus* L. × *Hordeum* L.

Typification: ×*Elymordeum dutillyanum* Lepage (type hybrid); Quebec: James Bay, Vieux-Comptoir [Old Factory], *Lepage 32060, July 29, 1954* (holotype, CAN!; isotypes, DAO!, RIM); from same tuft as holotype, *Dutilly, Lepage, and Duman 32996, Sept. 6, 1954* (clonotypes and paratypes, DAO!, CAN!, RIM).

Gramen hybridum atque intermedium inter *Elymum* et *Hordeum*; spiculae (2)–3–(7) in nodo; flores (1)–2–3–(4) in spicula, omnes steriles, i.e. semina non formantes; aristae et appendices spicae summae inter parentes intermediae.

Artificial and natural ×*Elymordeum* hybrids have the following characteristics: spikelets (two)–three–(seven) at the rachis nodes with (one)–two–three–(four) flowers per spikelet, completely sterile, i.e. not forming seeds; spikelets somewhat heterospiculate, with usually very short pedicels on the lateral spikelets and slightly larger sessile or nearly sessile central spikelets; spike tips variable, usually a single large or reduced terminal spikelet and sometimes the spike tip terminating in a short rudimentary structure; awns intermediate in length between the *Hordeum* and *Elymus* parents. ×*Elymordeum* hybrids are thus more or less intermediate in characteristics between the parental genera.

Some characteristics of ×*Elymordeum* hybrids are strictly related to the generic characters of the parents. Other characteristics of the hybrids depend upon the section, species, variety, or form of each parent, for example, an awnless or awned *Elymus* or a short-awned or long-awned *Hordeum*.

The characteristics of the parental genera are:
Elymus: spikelets two–six, rarely more, at the rachis nodes with three–six flowers per spikelet; all spikelets fertile and isospiculate, sessile or with very short pedicels; terminal spikelet present.
Hordeum: Three spikelets per rachis node with each spikelet one-flowered and usually with a naked rachilla; heterospiculate with central spikelets fertile, the laterals sterile and reduced to glumes but sometimes with anthers, except in *H. vulgare* L., which is isospiculate and all three spikelets fertile (i.e. form seeds); no terminal spikelet present, the subterminal spikelets gradually smaller and near the apex forming numerous small rudimentary structures and the rachis tip always terminating in a small rudimentary structure, the apical structures usually without pigmentation. It should be noted that careful dissections are needed to observe the rachis-tip structures.

I have used the name ×*Elymordeum* Lepage for hybrids of *Elymus* × *Hordeum* because it appears to be the only validly published name for these hybrids. The only taxon that might be, but has not proved to be, *Elymus* × *Hordeum* is *Elymus europaeus* L.; it has also been called *Hordeum europaeum* (L.) All., *Cuviera europaea* (L.) Koeler, and *Hordelymus europaeus* (L.) Jessen ex Harz. The correct generic name, other than *Hordeum* L. and *Elymus* L., is *Hordelymus* Jessen ex Harz. *Cuviera* Koeler is illegitimate since it is a homonym of the conserved *Cuviera* DC. listed by Lanjouw *et al.* (16). I have rejected ×*Hordelymus* as a name for hybrids of *Hordeum* × *Elymus* (or *Elymus* × *Hordeum*) on taxonomic grounds and my opinion is also based on morphological and genetic data. Mansfeld, in an editorial footnote by Stubbe in Zizin and Petrowa (27), rejected ×*Hordelymus* sensu Bacht. et Dar. as a name for hybrids of *Hordeum* × *Elymus* on taxonomic grounds.

The characteristics of *Hordelymus europaeus* (L.) Jessen ex Harz are: usually three spikelets at each rachis node with the spikelets one–(two)-flowered; spikelets with short pedicels of equal length, often isospiculate but sometimes the central spikelets slightly larger than the laterals, and often the spikelets heterospiculate with the central spikelets reduced to empty glumes and rudimentary parts and the lateral spikelets well developed and bearing seeds; one terminal spikelet present at the tip of each spike.

Hubbard (14) described *Hordelymus europaeus* and stated that it seeds "...profusely, the seeds germinating in the autumn and flowering the following year. As it produces so few vegetative shoots, it rarely lasts more than two or three years." He also wrote that "on the Chilterns it is sometimes locally quite abundant, especially in those beech-woods with undergrowth, and after a good seeding year."

Because of the rather unusual combination of morphological characteristics, *Hordelymus europaeus* has proved difficult for botanists to classify. Some taxonomists have thought that the characteristics were a combination of *Hordeum* and *Elymus* characters, but my analysis indicates that *Hordelymus europaeus* is not the result of the crossing of a species of *Hordeum* with a species of *Elymus*.

The most significant characteristics of *Hordelymus europaeus* are the presence of the single terminal spikelet per spike, the usually one-flowered, sometimes two-flowered spikelets, the isospiculate condition of the short-pedicelled spikelets or if heterospiculate, the central spikelets reduced, not the laterals as in *Hordeum*, and the fact that abundant seeds are produced.

× *Elymordeum* differs from *Hordelymus* Jessen ex Harz in the spike tip, usually with a single large or reduced terminal spikelet and sometimes the spike tip terminating in a small very short rudimentary rachis tip with one or more empty glumes or small rudimentay spikelets, in the spikelets (one)–two–three–(four)-flowered and the spikelets somewhat heterospiculate with usually very short pedicels on the lateral spikelets and slightly larger sessile or nearly sessile central spikelets; and the spikelets completely sterile, i.e. never forming seeds.

× *Elymordeum* Hybrids

Index of × *Elymordeum Hybrids*

In the present paper, the symbol ! is used when the hybrid parentage has been experimentally confirmed. The symbols of Lanjouw and Stafleu (17) have been used in designating the herbaria where specimens are permanently deposited.

1. × *Elymordeum dutillyanum* (*Elymus mollis* subsp. *mollis* × *Hordeum jubatum*).
2. × *Elymordeum schaackianum* (*Elymus hirsutus* × *Hordeum brachyantherum*).
3. × *Elymordeum piperi* (*Hordeum jubatum* × *Elymus triticoides*).
4. × *Elymordeum dakotense* (*Hordeum jubatum* × *Elymus canadensis*).
5. !× *Elymordeum triploideum* (*Hordeum distichon* × *Elymus racemosus* var. *racemosus*).
6. !× *Elymordeum stebbinsianum* (*Hordeum brachyantherum* × *Elymus glaucus*).
7. !× *Elymordeum berkeleyanum* (*Hordeum brachyantherum* × *Elymus condensatus*).
8. !× *Elymordeum montanense* (*Elymus virginicus* × *Hordeum jubatum*).
   8*a*. With glabrous to sparsely scabrous lemma backs . . . nm. *montanense*.
   8*b*. With pubescent lemma backs . . . nm. *pubescens*.

1. × ELYMORDEUM DUTILLYANUM Lepage, Nat. Can. 84:97–102. 1957.

Formula: *Elymus mollis* Trin. subsp. *mollis* × *Hordeum jubatum* L.

Typification: Quebec: James Bay, Vieux-Comptoir [Old Factory], touffes denses, haut du rivage, *Lepage 32060, July 29, 1954* (holotype, CAN!; isotypes, DAO!, RIM); from same tuft as holotype, *Dutilly, Lepage, and Duman 32996, Sept. 6, 1954* (clonotypes and paratypes, DAO!, CAN!, RIM).

Lepage (19) has given a detailed description of this natural hybrid and a table comparing the morphology of the hybrid and the supposed parents. I

have examined the specimens listed above and I think that Lepage has suggested the probable parentage of this hybrid. Lepage (in a letter) has stated that the specimens of *Lepage 32060* and *Dutilly et al. 32996* were collected from the same hybrid clump. An artificial hybrid of known parentage is needed to prove the parentage of this natural hybrid. Lepage (19) stated that *Elymus mollis* and *Hordeum jubatum* are the only species of these genera that occur on the east coast of James Bay and that they frequently grow in the same habitats. At present, ×*Elymordeum dutillyanum* is known only as a single hybrid plant at Old Factory, James Bay. It may occur rarely in other areas where *E. mollis* and *H. jubatum* grow near one another. There are several potential stations, for example, along the St. Lawrence River below Quebec City, the beaches of New Brunswick, Nova Scotia, and Prince Edward Island, and several localities on the western and southern shores of Alaska.

An examination of the morphology of the type specimens of ×*Elymordeum dutillyanum* certainly indicates that the plant is a hybrid. The pubescence of the awns and lemmas of the hybrid resembles that of *E. mollis* subsp. *mollis* and the hybrid has well-developed rhizomes that are less coarse than those of *E. mollis*. The long awns of the glumes and lemmas, the dense spikes, the spike-tip structures, and the tendency for the spikelets to occur in threes at the rachis nodes of the hybrid spikes show the influence of the *Hordeum jubatum* parent. In the hybrid, there are one–three flowers per spikelet, which is characteristic of ×*Elymordeum* hybrids. I examined several anthers of *Lepage 32060* (DAO!); the anthers were narrow, the pollen remained inside the anther walls and the pollen was completely bad when tested in iodine–potassium iodide solution. The mature specimens of *Dutilly et al. 32996* (DAO!, CAN!) did not set any seed but there is an ergot sclerotium in one of the spikes (CAN!).

2. ×ELYMORDEUM **schaackianum** hybr. nov., inter parentes intermedium; spiculae 2–3 in nodo; flores 1–2 in spicula; spiculae laterales pedicellibus brevibus; spiculae centrales sessiles vel pedicellibus brevibus; glumae angustae et crassae; lemmata ad dorsum glabra, marginibus ad apiceum breviter pubescentibus.

Formula: *Elymus hirsutus* Presl × *Hordeum brachyantherum* Nevski.

Typification: Alaska: Aleutian Islands: Attu Island, vicinity of Massacre Bay, in rich soil on primitive village site near beach, behind navy bakery, *Lt. G. B. Van Schaack 849, Aug. 10, 1945* (holotype, DAO!, isotype, MO); Attu Island: Sarana Valley, near the shore of Lake Nicholas, single culms, blades flat, in sandy loam, *G. W. Soule, M.D., 521, September 2, 1945* (paratypes, DAO!, MO).

The specimens of this hybrid are intermediate between the supposed parents. There are two–three spikelets at the rachis nodes and one–two flowers per spikelet. The lateral spikelets have short pedicels and the central spikelets are sessile or nearly sessile. The glumes are narrow and coarse and the lemma backs are glabrous with the margins short pubescent towards the apices.

Dr. Van Schaack kindly sent the two hybrid specimens and specimens of the supposed parents for deposit in the Divisional Herbarium (DAO) when he learned of my interest in × *Elymordeum* hybrids. In a letter, Van Schaack wrote: "I feel sure that Nos. 849 and 521 represent genuine hybrids of the two species represented in 675 and 516. *Hordeum brachyantherum* is moderately common on Attu Island, having been collected by Dr. Soule and myself in quite a number of places in the eastern end of the island. *Elymus hirsutus*, on the other hand, although it may be common, we found only in Sarana Valley. The hybrid was found just twice, once in Sarana Valley (Soule 521), and once on the other side of the mountain in Massacre Bay Valley (Van Schaack 849). As I remember it, this last stand consisted of a single thick colony which may have been more than one plant but which may have been only one plant several years old."

The specimen of *Van Schaack 675* is *Hordeum brachyantherum* Nevski and was collected on Attu Island: Chicagof Harbor: Attu Village site. The specimen of *Soule 516* is *Elymus hirsutus* Presl and was collected on Attu Island: Sarana Valley. The specimen of *Van Schaack 849*, the holotype of × *Elymordeum schaackianum*, has three spikelets at the rachis nodes and two flowers per spikelet. The second flower in each spikelet is rudimentary. The paratype, *Soule 521*, has shorter spikes, two spikelets at the rachis nodes, and one–two flowers per spikelet; the second flower is rudimentary or there is a naked rachilla. Both specimens have short pubescence on the margins of the lemma apices which shows the influence of the *Elymus hirsutus* parent.

I examined several anthers from *Van Schaack 849* and *Soule 521*. In both specimens, the pollen remained inside the anther walls. When tested in iodine – potassium iodide solution, the pollen was completely bad. The condition of the pollen indicates hybridity and the morphological characteristics of the type specimens are evidence that Dr. Van Schaack has discovered the probable parentage of this hybrid.

3. × ELYMORDEUM **piperi** hybr. nov., spiculae 3 in nodo; flores 2–(3) in spicula; spiculae sessiles vel pedicellibus brevissimis; glumae setiformes; lemmatibus glabris.

Formula: *Hordeum jubatum* L. × *Elymus triticoides* Buckl.

Typification: Oregon: Hot Lake, *C. V. Piper s.n., June 22, 1921* (holotype, US 1107887!; photograph, DAO!).

This specimen is intermediate morphologically between the supposed parents. In the hybrid, there are three spikelets at the rachis nodes and two–(three) flowers per spikelet. The spikelets are sessile or with very short pedicels. The glumes are setiform and the lemmas are glabrous. The three spike tips of the holotype exhibit reduced or small rudimentary spikelets intermediate between the terminal spikelets of *Elymus* and the reduced or rudimentary terminal spikelets of *Hordeum*.

Covas (8) thought that the parentage of the holotype was probably *Hordeum jubatum* L. × *Elymus triticoides* Buckl. The morphology of the holotype

indicates that Covas suggested the probable parentage of this hybrid. I examined several of the narrow anthers of the holotype. The pollen remained inside the anther walls and the pollen was completely bad when tested in iodine – potassium iodide solution.

4. × ELYMORDEUM **dakotense** hybr. nov., spiculae 2–(3) in nodo; flores 2–(3)–(4) in spicula; glumae crassae paullum dilatatae ad basas; glummae et lemmata longiaristata; aristae ad 3.2–4.3 cm. longae; lemmata ad dorsum breviter pubescentia.

Formula: *Hordeum jubatum* L. × *Elymus canadensis* L.

Typification: South Dakota: Brookings, *J. T. Sarvis No. 3, 8/11* (holotype, US 730679!; photograph, DAO!).

Covas (8) thought that this specimen was probably a hybrid of *Hordeum jubatum* L. × *Elymus canadensis* L. I have examined this hybrid carefully and the suggested parentage would explain the distinctive characteristics of the specimen. An artificial hybrid is needed to confirm the parentage. The holotype has strong characteristics that distinguish it from the other × *Elymordeum* hybrids, although the pubescence of the lemma backs resembles that of the lemma backs of ×*Elymordeum montanense* nm. *pubescens*. The holotype of ×*Elymordeum dakotense* has two–(three) spikelets at the rachis nodes and two–(three)–(four) flowers per spikelet. The glumes are coarse and slightly broadened towards the bases. The glumes and lemmas are long-awned with awns up to 3.2–4.3 cm. long. The lemma backs are short-pubescent. I was unable to find any anthers in several spikelets that were examined.

5. !× ELYMORDEUM **triploideum** hybr. nov., based on the description and figures of Bachtjeev and Darevskaja in Botan. Zhurn. Akad. Nauk S.S.S.R. 35:188–191 and Figs. 1–3. 1950. In Russian.

×*Hordelymus zizinii* Bacht. et Dar., l.c.:191. Nomen invalidum (no Latin description).

×*Elyhordeum zizinii* (Bacht. et Dar.) Mansf. ex Stubbe apud Zizin et Petrowa in Der Züchter. 25:164. 1955. Nomen invalidum (no Latin description) based on ×*Hordelymus zizinii* Bacht. et Dar., nomen invalidum (no Latin description).

Formula: *Hordeum distichon* L. cultivar 'Wiener' (♀) $n=7$ × *Elymus racemosus* Lam. var. *racemosus* (♂) $n=14$.

Typification: Artificial hybrid grown at The Institute for Grain Husbandry, Nemchinovka, Moscow Region, USSR, and specimens presumably preserved in their herbarium; description and Figs. 1–3 of Bachtjeev and Darevskaja (l.c.); see article 10 of Lanjouw *et al.* (16).

I have based the following Latin description of !×*Elymordeum triploideum* on the detailed description of Bachtjeev and Darevskaja (3): spiculae 6–7–(2–3) in nodo; flores 3–2 in spicula, steriles; glumis lineari-lanceolatis, breviaristatis, glabris; lemmatibus breviaristatis 7–nervis, densiter breviterque pubescentibus; $2n=21$. The spikelets were six–seven–(two–three) at the rachis nodes

and the spikelets were three–two-flowered and sterile. The glumes were linear-lanceolate, short-awned, and glabrous. The lemmas were short-awned and seven-nerved with dense short pubescence. The somatic chromosome number was $2n=21$ and was recorded by Bachtjeev and Darevskaja (2) from cytological studies by V. A. Arnoldi who also made chromosome counts of the parental plants.

In 1943, Bachtjeev and Darevskaja (2) obtained a single hybrid seed from the cross, two-rowed barley, *Hordeum distichon* L. (var. *nutans* Schübl.) cultivar 'Wiener' (♀) $n=7$ × *Elymus racemosus* Lam. var. *racemosus* (♂) $n=14$. Bowden (6) showed that *E. racemosus* Lam. was an earlier name for *E. giganteus* Vahl, the name used for the pollen parent by Bachtjeev and Darevskaja (2, 3). A description and figure of *Hordeum distichon* L. var. *nutans* Schübl. was given by Hegi (11). The characteristics of each of the parental plants were described by Bachtjeev and Darevskaja (3). The triploid hybrid was grown in 1944 and produced vigorous growth and profuse tillers. A large clone was developed from the original single plant. The authors noted that the vegetative plant showed a dominance of the paternal *Elymus* characteristics. The hybrid clone produced one spike in 1945 that was illustrated in Fig. 3 of Bachtjeev and Darevskaja (3). The spike exhibited a dominance of the paternal *Elymus* characteristics but showed the influence of the maternal *Hordeum* characters. The dominance of the paternal characteristics may have been, at least in part, a quantitative effect of the 14 *Elymus* chromosomes over the seven *Hordeum* chromosomes in each hybrid nucleus. Bachtjeev and Palamarčuk (4) investigated the cytology of !× *Elymordeum triploideum*. They observed numerous meiotic irregularities and from two to six bivalents and 17 to nine univalents at metaphase I. When mature, the pollen was completely bad.

6. !× ELYMORDEUM **stebbinsianum** hybr. nov., inter parentes stans et pro vegetativis et pro floralibus; spiculae fere semper ternae in nodo; flores 1–2–(3) in spicula, steriles; spiculae laterales pedicellibus brevibus; spiculae centrales sessiles vel pedicellibus brevibus; glumae aristatae ad basas dilatatae; paleae sicut *Elymi*.

Formula: *Hordeum brachyantherum* Nevski (♀), parent plant, 401–3, ex Berkeley, California, herb. no. 2747 × *Elymus glaucus* Buckl. (♂), pollen parent plant, 404–2, ex Santa Paula, California, herb. no. 2866.

Typification: Artificial hybrid No. 558–1 described by Stebbins *et al.* in Amer. J. Bot., 33:579–586 and Figs. 1D, 2D, 3F, and 3G. 1946. *G. L. Stebbins, Jr., August 7, 1945* (holotype, UC!; photograph, DAO!).

Artificial and natural hybrids of this parentage were reported by Stebbins *et al.* (26) in 1946. The *Hordeum brachyantherum* parent was originally identified as *H. nodosum* L. The following description of !× *Elymordeum stebbinsianum* is based partly on the description of Stebbins *et al.* (26) and partly on my examinations of the holotype and paratypes (cited below). These hybrids are intermediate between the parents in both vegetative and floral characteristics. There are usually three spikelets at the rachis nodes and

one–two–(three) flowers per spikelet. The lateral spikelets have very short pedicels and the central spikelets are sessile or nearly sessile. The glumes are awned with a broadened basal portion and the paleas resemble those of *Elymus*. Meiosis was very irregular in the artificial hybrid, the pollen was bad, and the plants never set seed (Stebbins *et al.*, l.c.). I examined one narrow anther from the holotype of !×*Elymordeum stebbinsianum*. The pollen remained inside the anther walls and the pollen was completely bad when tested in iodine – potassium iodide solution.

Specimens Examined of !× Elymordeum stebbinsianum

Holotype: Artificial hybrid No. 558–1, produced by Dr. G. L. Stebbins, Jr., *et al.*, Division of Genetics, University of California, Berkeley, California, hybridized in May, 1944, and specimen made by *G. L. Stebbins, Jr., August 7, 1945* (UC). Natural hybrids (paratypes): CALIFORNIA: Tuolumne County: Yosemite National Park, Cottonwood Meadows, moist open ground, with its parents, only one plant seen, *G. L. Stebbins, Jr., 3529, August 24, 1945* (UC); Contra Costa County: 2 miles east of El Cerrito, near Mira Vista Country Club, open grassy hills in heavy clay soil, *G. L. Stebbins, Jr., 3659, June 9, 1946* (UC). OREGON: West meadow of the Dead Indian Summit of the Cascades Mts., *Wm. C. Cusick 2958* (US); Fremont National Forest, rich loam, 7000 alt., *Jay Billings 2460* (US). WASHINGTON: W. Klickitat County: Falcon Valley: border of a meadow near Fulda, *W. N. Suksdorf 5194*, two sheets (US); one sheet (MO).

7. !× ELYMORDEUM **berkeleyanum** hybr. nov., spiculae subsessiles saepius 3–4 in nodo; flores 2–3 in spicula; glumae et lemmata breviaristata; lemmata ad dorsum glabra.

Formula: *Hordeum brachyantherum* Nevski (♀), parent plant, 568–5, ex Almonte, Marin County, California, × *Elymus condensatus* Presl (♂), pollen parent plant, 2859 ex Gorman, Los Angeles County, California.

Typification: Artificial hybrid No. 621–3 produced by Dr. G. L. Stebbins, Jr., at Berkeley, California. *G. L. Stebbins, Jr., June 14, 1947* (holotype, UC!; photograph, DAO!).

The specimen of the holotype of this hybrid has coarse culms. There are usually three–four nearly sessile spikelets at the rachis nodes and the spikelets are two–three-flowered. The glumes and lemmas have short awns and the lemma backs are glabrous. I examined two of the narrow anthers. The pollen remained inside the anther walls and the pollen was completely bad when tested in iodine – potassium iodide solution.

8. !× ELYMORDEUM **montanense** (Scribn. in Beal, pro sp.) comb. nov., based on *Hordeum montanense* Scribn. in Beal, Grasses N. Amer. 2:644. 1896.

Formula: *Elymus virginicus* L. × *Hordeum jubatum* L.

Typification: Montana, *Scribner 430* (holotype, labelled type by Scribner, US!; photograph, DAO!; isotype, GH!).

8*a*. !×ELYMORDEUM MONTANENSE (Scribn. in Beal) comb. nov. nm. MONTANENSE; *Hordeum montanense* Scribn. in Beal sensu stricto.

Hordeum pammeli Scribn. and Ball in Pammel, Ball and Scribner, Iowa Geol. Survey Sup. Rept., 1903. The Grasses of Iowa, part II: 335–337 and Fig. 237. 1905. Type: Dakota City, Iowa, *L. H. Pammell 3824* (US!).

Descriptions applying to !× *Elymordeum montanense* nm. *montanense* have been given by Scribner in Beal (5), Scribner and Ball (22), Hitchcock (13), and Fernald (10). The significant characteristics are: natural and artificial hybrids from the cross, *Elymus virginicus* × *Hordeum jubatum*; more or less intermediate between the parents; spikelets three at each rachis node with two–(three)–(four) flowers in each spikelet, the second, third, and fourth flowers, if present, sometimes reduced to empty glumes or to naked rachillae; stamens and ovules present at least in the first flowers of the central and lateral spikelets; completely sterile, i.e. never forming seeds; spikelets somewhat heterospiculate, with very short pedicels on the lateral spikelets and often slightly larger sessile central spikelets; spike tips variable, usually a single large terminal spikelet, and sometimes the spike tip terminating in a small, very short rudimentary rachis tip with one or more empty glumes or small rudimentary spikelets; glumes subulate-attenuate, slightly broadened above the bases; glumes and lemmas with slender scabrous awns, the awns variable in length, usually the awns of the first lemmas of the middle spikelets longer; the longer awns in a group of spikelets, up to 1.3–1.6–2.5–3.0 cm. long; awns intermediate in length between the usually long awns of *Hordeum jubatum* and the short awns of *Elymus virginicus*; lemma backs with shiny surfaces, glabrous towards the bases, usually mostly glabrous but sometimes sparsely short-scabrous especially towards the apices and usually scabrous along the margins of the lemma apices.

In the specimen of *Breitung 428* from Tisdale, Saskatchewan, the lemma backs are more scabrous than those of the isotype (GH). The awns of *Scribner 430*, the holotype (US) and isotype (GH), are shorter than most specimens of nm. *montanense* and the longer awns in a group of spikelets measure up to 1.4 cm. long. Scribner in Beal (5) noted that the holotype of nm. *montanense* had shorter awns than *Scribner 429* (see below under nm. *pubescens*). The specimens collected by V. H. Chase at Princeville, Illinois, have the longest awns (up to 3.0 cm.) of any specimens of nm. *montanense* that I have examined.

The variation in awn length of various specimens of !× *Elymordeum montanense* nm. *montanense* is probably due to inherent differences in the parental plants that produced the different hybrids. In *Hordeum jubatum* L., Hitchcock (13) recognized the typical variety, var. *jubatum*, and a shorter-awned western variety, var. *caespitosum* (Scribn.) Hitchc. Most of the specimens cited below under nm. *montanense* probably had *H. jubatum* L. var. *jubatum*

FIGS. 1–2. Spikes of dried herbarium specimens. FIGS. 1*a* and 1*b*. Spikes of *Elymus virginicus* L. f. *virginicus*. FIG. 1*a*. *Erskine & Roland 1481* collected at Lower Onslow, Nova Scotia. FIG. 1*b*. B–241–S–S5, grown from a selfed seed from *Erskine & Roland 1481*, potted plant, *Wray M. Bowden, Aug. 1, 1956*; the seed parent of the artificial hybrid, B–300–S1. FIG. 2. Spike of *Hordeum jubatum* L. var. *jubatum*, 1517S2, potted plant, grown from seed collected by *Erskine & Roland 1482* at Route 4 bridge of Chigonois River and 1½ miles west of Lower Onslow; pollen parent of artificial hybrid, B–300–S1.

1A
1B
2
METRIC 1 2 3 4 5 6 7 8 9 10 11 12

PLATE II

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as one parent. I have already noted that the holotype and isotype of nm. *montanense* had shorter awns than the other specimens cited below. Perhaps *H. jubatum* var. *caespitosum* was one of the parents of the holotype and this resulted in the shorter awns of this specimen.

The shorter awn lengths of some hybrid specimens, particularly the holotype and isotype and Breitung's Tisdale collection, may also be due to differences in the awn lengths of the *Elymus virginicus* parent. Hitchcock (12) noted that the type specimen of *Elymus virginicus* L. had "glumes and lemmas smooth, the former about 1.5 cm. long, including the awn point of about 5 mm., the latter with awns 1 to 1.5 cm. long." Probably most of the specimens of nm. *montanense* cited below had *Elymus virginicus* L. f. *virginicus* as one parent. Breitung collected a specimen (DAO) of *E. virginicus* L. f. *submuticus* (Hook.) Pohl from east of the town of Tisdale, Saskatchewan, a year before he collected the hybrid specimen. The awns of Breitung's Tisdale specimen of nm. *montanense* are a little longer (up to 1.6–1.7 cm.) than those of the holotype and isotype of nm. *montanense* (up to 1.4 cm. long). The St. Jovite, Quebec, specimens of *Churchill s.n.* also had shorter awns (up to 1.3–1.6 cm. long) than specimens from Illinois and Nova Scotia (up to 1.6–2.3–2.5–3.0 cm.). The shorter awns of the first group of specimens may be the result of *E. virginicus* f. *submuticus* being one of the parents of these hybrids rather than *E. virginicus* f. *virginicus*.

As described in a later part of this paper, an artificial hybrid (Fig. 3) was produced by crossing *Elymus virginicus* L. f. *virginicus* (Fig. 1*b*) with *Hordeum jubatum* L. var. *jubatum* (Fig. 2). The parental plants were collected near Lower Onslow, Nova Scotia, and the artificial hybrid was identical with the natural hybrid (Fig. 4) from Lower Onslow. The artificial hybrid closely resembles the specimens of hybrids from Illinois. It is certain that the specimens cited below under !×*Elymordeum montanense* nm. *montanense* have originated from the cross, *Elymus virginicus* L. × *Hordeum jubatum* L. The hybrid specimens with the longer awns presumably have the same parentage as the artificial hybrid, i.e. *E. virginicus* L. f. *virginicus* × *H. jubatum* L. var. *jubatum*. Some of the natural hybrids with shorter awns, particularly the holotype and isotype of nm. *montanense* and the *Breitung* specimen from Saskatchewan may have had slightly different parentages, either *Hordeum jubatum* L. var. *caespitosum* (Scribn.) Hitchc. or an intermediate between var. *jubatum* and var. *caespitosum*, crossed with *Elymus virginicus* L. f. *submuticus* (Hook.) Pohl or an intermediate between f. *submuticus* and f. *virginicus*. *Hordeum jubatum* var. *caespitosum* is a western variety and *E. virginicus* f. *submuticus* occurs commonly in southern Manitoba, Saskatchewan, and adjacent States and rarely in the eastern part of the range of *E. virginicus*.

FIGS. 3–4. Spikes of dried specimens of !× *Elymordeum montanense* (Scribn. in Beal) comb. nov. nm. *montanense*. FIG. 3. Artificial F_1 hybrid, plot plant, B–300–S1, *Elymus virginicus* L. f. *virginicus* (♀), B–241–S–S5, a selfed seedling of *Erskine & Roland 1481*, × *Hordeum jubatum* L. var. *jubatum* (♂), 1517S2, grown from seed from *Erskine & Roland 1482*; collected in Arboretum plots by *Wray M. Bowden, November 7, 1956*. FIG. 4. Natural hybrid, *Erskine & Roland 1480* collected at Lower Onslow, Nova Scotia.

There are many different possible combinations of awn-length genes from the *Elymus* and *Hordeum* parents of !× *Elymordeum montanense.* It does not seem desirable to me to give new nothomorph names to each possible combination of awn-length genes and I have listed all these specimens under nm. *montanense.*

The type locality of !× *Elymordeum montanense* nm. *montanense* is Montana. The sheet of the holotype, *Scribner 430* (US!), was labelled type by Scribner and bears several notes concerning locality: "Montana" and "by a cold spring in the sun". Scribner in Beal (5) recorded the type locality as: "shaded borders, Hound Creek, and by springs near the head of Jan Coulie or Tiger Battle, Aug. 3, 1883." The sheet of the isotype, *Scribner 430* (GH!) bears a specimen that is identical with the holotype; the sheet has the words: "Montana, Head of Jan Coulie, Aug. 9, 1883." Stations for !× *Elymordeum montanense* have been recorded by Scribner and Ball (22), Hitchcock (13), Fernald (10), and Jones and Fuller (15). The natural hybrids, *Elymus virginicus* × *Hordeum jubatum*, occur rarely and sporadically in Montana, Saskatchewan, Wyoming, South Dakota, Iowa, Illinois, Quebec, and Nova Scotia. Each hybrid clone represents a separate intergeneric hybridization.

These natural hybrids are found occasionally in localities where populations of both of the parental species of *Elymus* and *Hordeum* occur in adjacent habitats. Possibly the hybrids may be found to occur in some of the adjacent states and provinces and there may be more stands in the areas where some hybrids have already been collected. The hybrids occur either in disturbed habitats or in natural locations where the parental plants grow near one another. Plants of *Elymus virginicus* usually grow in lightly shaded woods or along stream banks. Stands of *Hordeum jubatum* grow in open ground and often as weeds, either in fields or in dense bands along roadsides or disturbed soil. Hitchcock (13) stated that *Hordeum jubatum* has been introduced in the Eastern States. In southern Ontario, it often occurs as a weed along roadsides. There are many habitats where *H. jubatum* can grow in the prairie area from Illinois to Montana and Saskatchewan and the species is common along roadsides, fields, open ground, meadows, and waste lands.

Jones and Fuller (15) recorded some stations where V. H. Chase had collected specimens of !× *Elymordeum montanense* in three adjacent counties in northern Illinois. Plants of the parents are common in Illinois and occur in roadsides (both parents), woods (*E. virginicus*), and fields (*H. jubatum*). Plants of the hybrid have been found in disturbed habitats such as roadsides, dikeland banks, and moist ditches but some specimens have been collected in more natural habitats such as open woods, hillsides in partial shade, and damp prairies. On the field label of the hybrid specimen of *Erskine and Roland 1480* from Lower Onslow (Fig. 4), the collectors noted that the "colony" was about eight feet in diameter in 1952. Dore and Roland (9) recorded the condition of the "colony" 11 years before as "a single large colony". The clump probably grew from one hybrid seed and evidently is quite persistent and long lived. The artificial hybrid of *E. virginicus* × *H. jubatum* is also vigorous and has been grown under cultivation for several years without difficulty.

Hybrid specimens have not been reported from Indiana, Ohio, Michigan, Ontario, Pennsylvania, New York, or Maine. Until the presence or absence of hybrids in this area can be confirmed, it is not essential to try to explain the apparent absence of hybrids. If hybrids are absent in this area, the cause may be that plants of *Hordeum jubatum* have not occurred in habitats closely adjacent to plants of *Elymus virginicus*, so that hybrids could not be formed.

Specimens Examined of !× Elymordeum montanense nm. montanense

Natural hybrids: MONTANA: By a cold spring in the sun, *Scribner 430*, holotype, labelled type by *Scribner* (US); head of Jan Coulie, *Scribner 430, August 9, 1883* (isotype, GH). SASKATCHEWAN: Tisdale: river flat, tufted, some ergot present, distributed as *Elymus macounii, August J. Breitung 428* (CAN; DAO). IOWA: Dakota City, *L. H. Pammel 3824* (holotype of *Hordeum pammeli* Scribn. and Ball, US). WYOMING: moist sandy meadow, *David Griffiths 930* (US). SOUTH DAKOTA: Brookings Co.: Brookings, *E. H. Wilcox s.n., July 8, 1896* (US). ILLINOIS: Peoria County: Princeville, moist ditch, *Virginius H. Chase, June 30, 1907* (DAO; MT; GH); Stark County: Wady Petra, roadside, *Virginius H. Chase, July 28, 1907* (DAO; MT; GH). QUEBEC: Terrebonne County: St. Jovite, wasteground at the railroad station, identified by A. S. Hitchcock as *Hordeum pammeli* Scribn. and Ball, *J. R. Churchill s.n., Aug. 10, 1922* (US; GH). NOVA SCOTIA: Colchester County, Lower Onslow, a large colony near stream near dykelands, in rich soil, *A. E. Roland 41076, July 31, 1941* (DAO); same station, 2 miles east of Debert Turn-off and about 5 miles west of Truro, N.S., bank of Dikeland (fresh) Creek; one colony about 8 ft. in diameter with *Elymus virginicus* L., *D. Erskine and A. E. Roland 1480, August 9, 1952* (DAO with duplicates for distribution), Fig. 4; cultivated specimens collected from plants grown in the Arboretum plots from living plants of *Erskine and Roland 1480*, collected by *Wray M. Bowden, July 25, 1955, November 7, 1956*, and *July 4, 1957*, and a specimen grown from the same clump in a large box of soil, *Wray M. Bowden, June 28, 1955* (all DAO).

Artificial F_1 hybrid: Cyt. No. B–300–S1: parentage: *Elymus virginicus* L. f. *virginicus* (♀), B–241–S–S5, selfed seedling of *Erskine and Roland 1481*, originally collected at Lower Onslow, × *Hordeum jubatum* L. var. *jubatum* (♂), 1517S2, seedling from *Erskine and Roland 1482*, 1½ miles west of Lower Onslow, Nova Scotia; one clone from one artificial hybrid seed obtained in 1954 and grown in 1954–55. Preserved specimens: potted plant, greenhouse, *Wray M. Bowden, Feb. 2, 1955*; Arboretum plots, *Wray M. Bowden, July 25, 1955, July 4, 1957*, and *November 7, 1956* (Fig. 3); part of same clone grown in large box of soil in outside frames, *Wray M. Bowden, July 25, 1955* (all DAO).

8*b*. × ELYMORDEUM MONTANENSE (Scribn. in Beal) Bowden nm. **pubescens** nm. nov., *Hordeum montanense* Scribn. in Beal pro parte, holotypo excluso; a holotypo differt lemmatibus pubescentibus.

Formula: *Elymus virginicus* L. × *Hordeum jubatum* L.

Typification of nm. *pubescens*: MONTANA, *Scribner 429* (labelled 430, see note below), paratype of *Hordeum montanense* Scribn. in Beal, (holotype of nm. *pubescens*, US 1018844!; photograph DAO!); NORTH DAKOTA: Ward County 2 miles east of Minot, *Curtis Benton s.n., Oct. 21, 1952* (paratype, US!).

Scribner in Beal (5) cited two specimens when he first described *H. montanense*: "Montana, *Scribner 429, 430.* Shaded borders, Hound Creek, and by springs near the Head of Jan Coulie or Tiger Battle, Aug. 3, 1883. Number 430 has the shorter awns, and the floral glumes scabrid; number 429 has the longer awns and the floral glumes puberulent." No specimen labelled No. 429 could be found in the United States National Herbarium but there were two specimens labelled *Scribner 430.* One was marked "type" by Scribner and it is the holotype of nm. *montanense*; it has shorter awns than the other specimen, glabrous to sparsely scabrous lemmas and thus corresponds to No. 430 originally described by Scribner. On the sheet of the holotype, No. 25 was written in two places; one of the Nos. 25 had been deleted and No. 430 written in. The sheet also bore the words, "by a cold spring in the sun."

The second sheet was also labelled *Scribner 430* and on the label were the words "Elymus with No. 25, growing in a moist thicket in the shade." This second sheet also had the words "Montana" and "with pubescent spikelets". The awns of the specimen are much longer (up to 2.2–2.6 cm.) than those of the holotype of nm. *montanense* (up to 1.4 cm.) and the lemma backs are pubescent. The specimen fits the original description of *Scribner 429* that was given by Scribner in Beal (5). Since a specimen labelled *Scribner 429* cannot be found, I consider this second specimen bearing the words "with pubescent spikelets" to be the holotype of nm. *pubescens.*

The parentage of ×*Elymordeum montanense* nm. *pubescens* appears to be *Elymus virginicus* L. f. *hirsutiglumis* (Scribn.) Fern. × *Hordeum jubatum* L. var. *jubatum.* Forma *hirsutiglumis* differs from f. *virginicus* by its villous to hirsute lemmas and glumes. The pubescent lemma backs of nm. *pubescens* could be caused by the genes from f. *hirsutiglumis.*

Other Hybrids

Stebbins *et al.* (26) stated that the type of *Elymus aristatus* Merr. and three other specimens represented a different hybrid combination from the artificial and natural hybrid that I have called !×*Elymordeum stebbinsianum* (*H. brachyantherum* × *E. glaucus*). I have examined type specimens of *E. aristatus* Merr.: Oregon: Harney County: Silver Creek, *Cusick 2712* (holotype, US!; isotypes, MO!; UC!). The specimens appear to me to be intergeneric hybrids of *Sitanion* × *Elymus.* In 1945, Dr. Stebbins attached annotation labels to the isotype sheets and suggested that the parentage might be *Elymus cinereus* × *Sitanion hystrix.* Stebbins and Vaarama (25) found that hybrids of *Elymus glaucus* × *Sitanion jubatum* and *Elymus glaucus* × *Sitanion hystrix* resembled natural hybrids that had been called *Sitanion hanseni.*

I have examined another artificial hybrid made by Dr. Stebbins, who deposited a specimen in the Herbarium of The University of California at

Berkeley. The specimen is: Artificial hybrid No. 519–5, *Hordeum californicum* Covas and Stebbins, ♀ parent, 405–1 ex Santa Barbara, Santa Barbara Co., California × *Elymus junceus* Fisch, ♂ parent, 201 ex Russia via USSCS, N. G. Plains Sta., N.D., *G. L. Stebbins, Jr., June 27, 1947* (UC!; photograph, DAO!). I have not included this specimen in the list of ×*Elymordeum* hybrids in the present paper because the pollen parent, *Elymus junceus* Fisch., does not belong to either section *Elymus* or section *Psammelymus* of the genus *Elymus*. Nevski (21) transferred this species to the genus *Psathyrostachys* and it does differ from the *Elymus* species in the two sections mentioned above, particularly in the reduced number of flowers per spikelet. Until a revised classification of these genera is made, it seems best not to name this specimen as an ×*Elymordeum* hybrid.

Cytogenetics of !×*Elymordeum montanense* nm. *montanense*

Collections of the Natural Hybrid and Parents at Lower Onslow

In July, 1941, Dr. A. E. Roland discovered "a single large colony" of the hybrid at Lower Onslow, Nova Scotia (*Roland 41076*). The site, about 5 miles west of Truro, was visited by D. Erskine and A. E. Roland in August, 1952, and the stand was then about eight feet in diameter. Herbarium specimens and living plants of the natural hybrid, !×*Elymordeum montanense* nm. *montanense*, Cyt. No. 1515, *Erskine and Roland 1480* (Fig. 4) and of one of the supposed parents, *Elymus virginicus* L. f. *virginicus*, Cyt. No. 1516, *Erskine and Roland 1481* (Fig. 1*a*) were collected and shipped to Ottawa. The living plants thrived in the greenhouse and plots and the plants proved to be hardy during several winters. Herbarium specimens and ripe seeds of the other parent, *Hordeum jubatum* L. var. *jubatum*, were collected 1½ miles west of Lower Onslow where Route 4 crosses the Chigonois River. The specimens were: *Erskine and Roland 1482*; the seeds were planted under Cyt. No. 1517 (Fig. 2).

The Elymus virginicus Parent

Erskine and Roland noted that the plants of *Elymus virginicus* L. f. *virginicus* were "luxuriant along much of the brook" at the Lower Onslow site. Hitchcock (12) examined the type specimen of *Elymus virginicus* L., which is Savage Catalogue No. 100.5 (photograph, DAO!), and he noted that the specimen had smooth glumes and lemmas and short awns. On the sheet of the type specimen, Linnaeus had written "4 virginicus". It is the fourth species mentioned by Linnaeus (20) under *Elymus* in the Species Plantarum; the type locality was "Habitat in Virginia."

Mr. C. E. Hubbard of Kew kindly examined the type specimen of *Elymus virginicus* L. He wrote detailed notes that have been deposited in the type folder of *E. virginicus* in our herbarium (DAO). On the back of the type sheet, Linnaeus wrote "Elymus virginianus H. Ups." The specimens of *Erskine and Roland 1481* and B–241–S–S5, the Lower Onslow material that was used as the seed parent of the artificial hybrid, B–300–S1, resemble but are

not identical with the type of *E. virginicus*. The resemblance in most characteristics is so strong that I consider that the Lower Onslow specimens are f. *virginicus*. In his notes, Hubbard wrote that the adaxial leaf blade surfaces of the type were ". . . minutely and obscurely hairy, above on the nerves (upper surface), or only scabrid there (hairs more numerous where they have not been rubbed off by the fingers of botanists!) . . ." The Lower Onslow specimens have glabrous adaxial leaf blade surfaces. The glumes of the type have scabrid glume nerves, scabrid glume tips and margins, and scabrous awns. In the Lower Onslow specimens, there are short scabrous hairs on the margins towards the apices of the smooth glumes and the awns are scabrous; there are some short scabrous hairs on some of the glume nerves. The key to the varieties and forms of *Elymus virginicus* of Fernald (10) needs to be revised in light of Hubbard's notes on the type of *E. virginicus*.

The preserved specimens of the Lower Onslow material are: Nova Scotia: Colchester County: Lower Onslow, about 2 miles east of Debert Turn-off (5 miles west of Truro), banks of Dikeland Creek, very abundant, *D. Erskine and A. E. Roland 1481* (Fig. 1*a*); specimen of plant No. B–241–S–S5, used as seed parent of artificial hybrid, a selfed seedling grown from *Erskine and Roland 1481*, potted plant, frames, *Wray M. Bowden Aug. 1, 1956*, Fig. 1*b* (both DAO).

The Hordeum jubatum Parent

Dore and Roland (9) reported that *Hordeum jubatum* L. in Nova Scotia was "common throughout the province in pastures and around farmyards, but especially abundant along the salt marshes and on the dykes." In August, 1952, Erskine and Roland did not find plants of *Hordeum jubatum* at the Lower Onslow site of the natural hybrid, !× *Elymordeum montanense* nm. *montanense*, where plants of *Elymus virginicus* also grew abundantly. They travelled about 1½ miles west along Route 4 to the bridge of the Chigonois River to make collections of specimens, *Erskine and Roland 1482* and ripe seeds of *Hordeum jubatum* L. var. *jubatum*. The seeds were planted under Cyt. No. 1517 and the plants were grown to maturity in pots in the greenhouse and outside frames (Fig. 2). Hitchock (12) examined the type specimen of *Hordeum jubatum* L., which is Savage Catalogue No. 103.10 (photograph, DAO!). The sheet is labelled "6 K jubatum". It was the sixth species of *Hordeum* listed by Linnaeus (20) in the Species Plantarum and Linnaeus gave the type locality as "Habitat in Canada, Kalm". The Chigonois River specimens resemble the type closely and I consider that they belong to var. *jubatum*. The preserved specimens are: Original seed specimens: Nova Scotia: Colchester County: Chigonois River at Route 4 bridge, 1½ miles west of Lower Onslow and 6½ miles west of Truro, along dike, marsh, and roadside, tufted, *D. Erskine and A. E. Roland 1482*; pollen parent of the artificial hybrid, grown from seed from *Erskine and Roland 1482*, Cyt. No. 1517S2, potted plant, frames, *Wray M. Bowden, June 28, 1955* (DAO) Fig. 2.

Self-fertility of the Parental Plants

It was desirable to obtain some data on possible differences in sterility and fertility. After the Nova Scotian collections were well established in 1953, some selfing tests were made. One spike each of *Erskine and Roland 1481*, *Elymus virginicus*, and 1517S2, a *Hordeum jubatum* seedling grown from *Erskine and Roland 1482*, were bagged and self-pollinated. Spikes of both plants proved to be highly self-fertile. Some of the selfed seeds of the *Elymus virginicus* were planted and one of these, No. B–241–S–S5 (Fig. 1*b*), was later used as the seed parent of the artificial hybrid.

Production of the Artificial Hybrid

Several attempts were made to cross *Elymus virginicus* with *Hordeum jubatum*. Since the parental plants had proved to be self-fertile, it was necessary to emasculate and bag the spikes carefully. The spikelets of *E. virginicus* appeared to be the easier to emasculate and it was chosen as the seed parent. One cross-pollination attempt in June, 1953, gave negative results. On July 29, 1954, the cross was again attempted. The seed parent was B–241–S–S5 (Fig. 1*b*), a selfed seedling of *Elymus virginicus* L. f. *virginicus* of *Erskine and Roland 1481* from Lower Onslow. The pollen parent was 1517S2 (Fig. 2), which was a seedling of *Hordeum jubatum* L. var. *jubatum* grown from the original seed collection of *Erskine and Roland 1482* at the Chigonois River bridge.

Only one spike was emasculated, bagged, and cross-pollinated. One medium-sized seed was obtained from the cross and accessioned under No. B–300–S1. It was kept for a short time in an envelope in the laboratory at room temperature and in September, 1954, it was sown in a pot of finely sifted soil in the greenhouse. The hybrid seed germinated quickly and the growth of the hybrid seedling was rapid from the beginning. Extra hours of artificial light were given in the greenhouse and the hybrid seedling produced strong flowering culms with well-formed spikes in early February, 1955.

The artificial hybrid, B–300–S1, formed numerous vegetative shoots and the clump was broken up and propagated vegetatively. Part of the clump was planted in the Arboretum plots in the late spring of 1955 where it grew vigorously and produced large numbers of flowering culms. The rest of the clump was planted in a large box of soil near the greenhouse and it also thrived. Both clumps successfully survived the winters of 1955–56 and 1956–57. The clumps enlarged and produced many flowering culms during the growing season of 1956. The flowering culms were produced mostly in June and July but numerous spikes were also formed in the fall and some specimens were made in November (Fig. 3).

The artificial hybrid was morphologically identical with the natural hybrid (Fig. 4) from Lower Onslow, Nova Scotia. The parentage of the Lower Onslow hybrid is thus proved to be *Elymus virginicus* L. f. *virginicus* × *Hordeum jubatum* L. var. *jubatum*. It appears impossible to know if the maternal parent of the natural hybrid was the *Elymus* or the *Hordeum* parent. Since both species are self-fertile, it is noteworthy that the hybrids occur in

nature at so many different and widely separated localities. Some interesting questions can be asked. Were some spikes or spikelets of either the *Hordeum* or the *Elymus* pollen sterile? Was a hybrid seed occasionally produced by cross-pollination because there were many more plants of one parental species in a particular locality?

Sterility of the Artificial and Natural Hybrids

A spike of the natural hybrid from Lower Onslow, *Erskine and Roland 1480*, was bagged in June, 1953. No seed was set. Numerous unbagged spikes were observed from 1953 to 1956 and they were always completely sterile. Numerous unbagged spikes of the artificial hybrid, B–300–S1, were examined during the growing seasons of 1955 and 1956. The spikes were always completely sterile, i.e. without seeds.

Pollen of the Natural and Artificial Hybrids

The specimen of *Roland 41076*, the original collection of the natural hybrid from Lower Onslow, was examined by Dr. R. J. Moore. He noted "pollen grains all empty, no starch nor protoplast, size somewhat variable, shape normal." In June, 1953, Dr. Moore examined a potted plant of *Erskine and Roland 1480*, the natural hybrid from Lower Onslow, and he observed that the pollen was empty and varied in size from (19.2)–ca. 30.0–(38.4) μ and that the anthers were thin. I examined a plot plant of the natural hybrid in June, 1955, and noted the small anthers. The pollen was completely bad when tested in iodine – potassium iodide solution and no starch was present. The pollen remained inside the anther walls.

A potted plant of the artificial hybrid, B–300–S1, was examined in July, 1955. The pollen was completely bad and all the pollen remained inside the anther walls. I also examined pollen from a greenhouse plant in January, 1955. The pollen was completely bad and there was no starch present in the pollen grains. There was a range of pollen size from (16.5)–43–46–(56.1) μ. Covas (8) noted that three specimens of !×*Elymordeum montanense* showed "100% pollen sterility and no seeds or developing ovaries were found."

Somatic Chromosome Number

The tetraploid number, $2n=28$, was found in the natural hybrid, !× *Elymordeum montanense* nm. *montanense*, *Erskine and Roland 1480*, collected at Lower Onslow; in the artificial hybrid, B–300–S1, and in plants of both of the parents, *Elymus virginicus* L. f. *virginicus*, *Erskine and Roland 1481* from Lower Onslow and *Hordeum jubatum* L. var. *jubatum*, Cyt. No. 1517, grown from seed from *Erskine and Roland 1482* from 1½ miles west of Lower Onslow, Nova Scotia.

Meiotic Chromosome Behavior of !×Elymordeum montanense nm. montanense

There were many meiotic irregularities in the pollen mother cells of the natural and artificial hybrids. It was rather difficult to find MI stages. On June 22, 1955, a plot plant of the natural hybrid, *Erskine and Roland 1480*, was studied and 10 pollen mother cells at MI showed the following numbers of

univalents and bivalents: 18 I + 5 II (two cells); 16 I + 6 II (three cells); 14 I + 7 II (two cells); 12 I + 8 II (three cells). The natural hybrid averaged 14.8 univalents and 6.6 bivalents per cell (average of 10 pollen mother cells).

On February 4 and February 7, 1955, anthers from central spikelets of a potted greenhouse plant of the artificial hybrid, B–300–S1, were examined and in MI stages, the following numbers of univalents, bivalents, and trivalents were observed: 26 I + 1 II (two cells); 24 I + 2 II (one cell); 22 I + 3 II (three cells); 20 I + 4 II (two cells); 18 I + 5 II (three cells); 16 I + 6 II (two cells); 14 I + 7 II (two cells); 10 I + 9 II (one cell); 18 I + 2 II + 2 III (one cell); and 15 I + 5 II + 1 III (three cells). The artificial hybrid averaged 18.45 univalents, 4.4 bivalents, and 0.25 trivalents per cell (average of 20 pollen mother cells from two slides). Other stages of meiosis in both the natural and artificial hybrids showed meiotic irregularities such as bridges, fragments, and micronuclei.

Boyle and Holmgren (7) studied meiosis in !×*Agrohordeum macounii* (Vasey) Lepage (*Agropyron trachycaulum* × *Hordeum jubatum*). The name × *Agrohordeum* was validly published by Rousseau (23) and Lepage (18) validly published the combination. Boyle and Holmgren (7) demonstrated that the parentage of !×*Agrohordeum macounii* is *Agropyron trachycaulum* × *Hordeum ubatum*. They studied meiosis in natural and artificial hybrids and reported a "high incidence of unpaired univalents and lagging chromosomes with resultant micronuclei. Eighty-nine percent of the cells observed contained at least 10 chromosomes associated in pairs or occasionally with one or two multivalents. They averaged 6.3 bivalents per cell." Parallel results were obtained in the study of meiosis in the natural and artificial hybrids of !× *Elymordeum montanense*. The higher irregularity of my artificial hybrid was probably because the anthers were from a greenhouse plant in February.

Discussion

Four putative and four proved hybrids of *Elymus* × *Hordeum* have been listed in this treatment of ×*Elymordeum* hybrids. One of the artificial hybrids, !×*E. triploideum*, was produced in Russia and the other artificial and natural hybrids were from North America. Seven of the parental species are native in North America: *Elymus hirsutus*, *E. triticoides*, *E. canadensis*, *E. glaucus*, *E. condensatus*, *E. virginicus*, and *Hordeum jubatum*. Two parental species occur in northeastern Asia and North America: *Elymus mollis* subsp. *mollis* and *Hordeum brachyantherum*. One parental species is native in central Asia, namely *Elymus racemosus*, and *Hordeum distichon* is a cultivated barley.

In the eight hybrids, *Hordeum brachyantherum* was one parent of three hybrids, *H. jubatum* was involved in four other hybrids, and *H. distichon* was one parent in another hybrid. Eight different species of *Elymus* were involved in the parentages of the eight ×*Elymordeum* hybrids. Bowden (6) has recently clarified two sectional names of the genus *Elymus* L. Of the eight parental species of *Elymus* that were parents of ×*Elymordeum* hybrids,

six species belong to section *Elymus* (syn.: section *Clinelymus* Griseb. in Ledeb.) that includes species with awned glumes and lemmas: *E. hirsutus*, *E. triticoides*, *E. canadensis*, *E. glaucus*, *E. condensatus*, and *E. virginicus*. Of these species, some are long-awned like the type species, *E. sibiricus* L., for example, *E. canadensis* L., and some are short-awned, for example, *E. virginicus*. Some future taxonomist may be able to show that these awned species should be grouped either as two sections of the genus *Elymus* or possibly two subsections of section *Elymus*. Two of the parental species of the ×*Elymordeum* hybrids belong to section *Psammelymus* Griseb. in Ledeb. that includes species with awnless glumes and lemmas: *E. mollis* subsp. *mollis* and *E. racemosus*.

More natural ×*Elymordeum* hybrids can be expected to occur rarely but sporadically in natural or disturbed habitats where plants of *Elymus* species grow near plants of *Hordeum* species. Only a very small number of plants of natural ×*Elymordeum* hybrids exist compared with the large numbers of individuals in populations of *Elymus* and *Hordeum* species. Additional artificial hybrids can be expected from further cross-pollination attempts. Several geneticists have told me that they knew of work presently in progress and more hybrids are being produced.

Artificial hybrids are needed to confirm the parentages of the four putative hybrids listed in the present paper. Two of these putative hybrids, ×*Elymordeum dutillyanum* and ×*E. schaackianum*, are thought to be hybrids on the evidence of the morphology of the supposed hybrids and parental plants, pollen sterility of the supposed hybrids, and the occurrence of only a few species of the parental genera in the areas where the supposed hybrids were collected. The parentages of the other two putative hybrids, ×*E. piperi* and ×*E. dakotense*, are based on the evidence of the morphology of the specimens of the supposed hybrids and supposed parental plants. The pollen was completely bad in ×*Elymordeum piperi* and no anthers could be found in ×*Elymordeum dakotense*. There are many species of *Elymus* and *Hordeum* in the areas where the last two hybrids were found and their parentages were more difficult to determine than the parentages of the first two hybrids.

Geneticists should preserve specimens of their artificial hybrids and the parental plants and deposit them in permanent herbaria. Such voucher specimens can then be used by later workers to check data published in scientific papers. Otherwise, later workers will be unable to use such data since there will be no way of identifying the materials except in those instances where detailed descriptions of the hybrids and parents have been recorded. In collecting natural hybrids, specimens of the supposed parental plants should also be collected near or at the same locality.

Of the eight ×*Elymordeum* hybrids listed in this paper, all except !×*E. triploideum* have involved only tetraploid ($2n=28$) species of *Elymus* and *Hordeum*, with the exception that I do not know the chromosome number of *Elymus hirsutus*. *Hordeum distichon* is the diploid ($2n=14$) involved in the parentage of !×*E. triploideum*. I have determined somatic chromosome

numbers for some collections belonging to the three species of *Hordeum* and six of the species of *Elymus* mentioned in the parentages of these eight × *Elymordeum* hybrids. Voucher specimens of the plants on which the chromosome numbers were determined are deposited in the Divisional Herbarium (DAO). Stebbins and Love (24) reported the tetraploid chromosome number, $2n = 28$, for *Elymus condensatus* Presl, No. 2859 ex Los Angeles Co., California, which was the pollen parent of !× *Elymordeum berkeleyanum*.

It is noteworthy that the anthers of ×*Elymordeum* F_1 hybrids always have bad pollen and that the spikes never set seed. In plants of the parental species of *Elymus* and *Hordeum*, the anthers normally dehisce and shed pollen. There are high percentages of pollen with starchy contents when tested in iodine – potassium iodide solution and the plants of the parental species normally set large amounts of viable seed. Ashman and Boyle (1) have suggested that meiotic irregularities are mainly responsible for the sterility of certain hybrids but pointed out that "unfavorable genetic factors may be another source of reduced fertility." They produced a partly fertile colchicine-induced octoploid strain of !×*Agrohordeum macounii*.

In concluding this paper, I shall briefly discuss the value of naming hybrids. In Appendix I of the Code (16), it is stated that hybrids can be "designated by a formula and, whenever it seems useful or necessary, by a name." In verbal discussions and correspondence with eight North American botanists, three did not approve of the practice of naming hybrids while five considered that naming hybrids will advance scientific knowledge.

The three botanists who did not approve naming hybrids gave several reasons: (1) ". . . I do not feel that much is to be gained from the practice of making up special names . . ."; (2) "Hybrids are freaks of nature and shouldn't be named."; (3) ". . . one less [if not named] epithet for the confused nontaxonomist to remember . . ."; (4) "The formula is best and is adequate and informative." Nos. 1 and 2 are personal opinions and Nos. 3 and 4 are discussed below.

In discussions with five other botanists, numerous points were in favor of naming hybrids. In the author's opinion, the naming of hybrids should advance scientific knowledge of plants and help to bring about a more systematic treatment of specimens in herbaria, in monographs, and in floras. The following points can be mentioned:

1. The formulae of hybrid parentages do not constitute names for hybrids and intergeneric hybrids lack proper names until designated by prescribed taxonomic methods. If the correct name of a hybrid is not used, the specimen sheets of the hybrid may bear incorrect names; for example, the numerous sheets of the proved hybrid, !×*Agrohordeum macounii* (Vasey) Lepage, are often labelled *Elymus macounii* Vasey but the taxon cannot be placed in *Elymus* because the specimens are hybrids of *Agropyron* × *Hordeum*.

2. If hybrid names are used, it is easier to file and locate specimens of hybrids in the herbarium and in floral lists.

3. In the text of scientific papers, hybrid names are often simpler to handle than formulae.

4. If hybrids are named, then they will be indexed in The Gray Index and Index Kewensis and botanists can locate the references much more easily than when formulae are used, since formulae are not indexed unless the hybrid has been named.

5. It is obvious that the formulae of the parentages of hybrids are very useful. But they have their limitations. Sometimes the names of species or the identifications of parents stated in a hybrid formula undergo changes so that the formula is not always stable. Once a name is used for a particular hybrid specimen, the name applies to that specimen no matter what nomenclatural or taxonomic changes may affect the names of the parents. If later workers come to different conclusions concerning the nomenclature or taxonomy of the parents, it may be necessary to transfer a hybrid name to a different hybrid genus, or to the rank of nothomorph of another hybrid if the same species parents are involved in two different hybrids.

6. From a pedagogical viewpoint, each hybrid involves a new concept and providing a name is the initial procedure in investigating and developing each new concept. The names of the hybrids become convenient words by which one can refer to the concepts and specimens.

7. Many artificial interspecific and intergeneric hybrids have been developed by plant breeders. These hybrids have recently assumed much greater importance in plant-breeding programs because of the use of colchicine to induce fertile polyploids. Names for hybrids and their derivatives will be very useful to plant scientists when they classify their material or list data concerning hybrid plants. Of course some judgment should be used as to how many and what hybrids should be named. The main criterion as to whether a hybrid should be named is whether or not the name will be useful to taxonomists, plant breeders, or horticulturists.

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THE PEACH REPLANT PROBLEM IN ONTARIO

V. THE RELATION OF PARASITIC NEMATODES TO REGIONAL DIFFERENCES IN SEVERITY OF PEACH REPLANT FAILURE[1]

W. B. MOUNTAIN AND H. R. BOYCE[2]

Abstract

Peach production in Ontario is largely restricted to the Niagara Peninsula and Essex County, areas that are separated by some 200 miles but have a similar climate. The peach replant problem has been much more serious in Essex County than in the Niagara Peninsula. A survey of mature peach orchards showed that *Pratylenchus penetrans* (Cobb, 1917) Sher & Allen, 1953, is considerably more prevalent in peach soils in Essex County than in the Niagara Peninsula. In both areas, orchards that had a previous history of the replant problem had three to four times greater soil population of *P. penetrans* than those with no such history. Soils of finer texture were shown to limit the populations of *P. penetrans*, and the relatively low numbers of this nematode in the Niagara Peninsula appear to result from the influence of the finer soils prevailing in that region. The effect of different soil-particle sizes on the build-up of *P. penetrans* may explain the distribution of the peach replant problem in Ontario.

Introduction

Several workers have reported on the peach replant problem in Ontario. Koch (2) discussed the symptomatology and distribution of the problem and reviewed some of the pertinent literature. Patrick (5) showed that microbial action on the amygdalin fraction of peach root residues resulted in toxicity to living peach roots. Ward and Durkee (8) demonstrated that the highest concentrations of amygdalin occur in the bark of peach roots. Wensley (9) found 14 species of fungi to be facultative parasites on the roots of peach seedlings in the greenhouse. The present authors have studied the relation between numbers of plant parasitic nematodes and intensity of peach replant failure in the two main areas of Ontario in which peaches are grown.

Regional Differences in Severity of the Replant Problem in Ontario

The larger of the peach-growing areas is the Niagara Peninsula, where there are approximately 11,600 acres of peaches; the other area is Essex County with approximately 1800 acres. Other very small peach-growing areas are scattered throughout southwestern Ontario.

According to the survey reported by Koch (2), 65% of questionnaires returned from Essex County indicated that replanted trees were killed or their growth was moderately to severely retarded. During the present investigation the authors observed that approximately 50% of the growers in Essex County experienced marked difficulty in reestablishing peach orchards on the finer-textured soils.

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Joint contribution from the Botany and Plant Pathology Division (No. 1644) and the Entomology Division (No. 3702), Science Service, Canada Department of Agriculture, Ottawa, Ontario.

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In the Niagara Peninsula the problem appears to be less serious. An appraisal of the severity of the problem in the Niagara Peninsula was requested from the staff of the Vineland Horticultural Experiment Station. Dr. J. F. Brown replied as follows: "It seems to be the case that the replant problem with peach is of minor importance here. This opinion is based on the fact that very few growers have complained to us about having difficulty in replanting peaches and on the fact that our staff members and the horticultural fieldmen located here have not run into a single really serious replant problem despite the fact that they visit a good many growers in the course of a season." Koch (2) states that "Aboveground symptoms of affected peach replants in Ontario are usually in the form of retarded growth . . ." Therefore, it would be very difficult to determine, without accurate measurements, whether the problem exists in an area. On the other hand, major differences in the severity of the problem should be apparent to trained observers. It is evident that differences do exist between the two areas. The authors have spent considerable time in studying regional differences in the severity of this disease because they believe these may be more important than at first appears.

The two discrete peach-growing areas are some 200 miles apart, have similar climates, and within them the same varieties are grown and similar cultural practices prevail. Yet, in Essex County, difficulty in reestablishing peach has occurred in over 50% of the orchards, whereas in the Niagara district such difficulties, according to Brown, have occurred in considerably less than 5% of the orchards. It appeared that such regional differences in the severity of the disease might provide a key to its solution, and that any explanation of the cause should also explain the distribution of replant failure.

Distribution of Plant Parasitic Nematodes in Relation to Occurrence of the Problem

As Koch (2) has mentioned, the peach replant problem is basically a root-rot disease and, as such, the factor or factors responsible for the condition are essentially soil-borne. These factors apparently build up in the soil during the life of the preceding peach orchard. Furthermore, the unequal intensity of the disease, as indicated in the preceding section, suggests that the soil-borne factors responsible for the trouble are likely to be unevenly distributed. In accordance with this reasoning a survey of mature peach orchards was undertaken to determine whether a characteristic parasitic nematode fauna was associated with the roots of peach trees, and how consistently such a fauna occurs in the two areas.

During 1954 and 1955, soil samples from 167 peach orchards were analyzed qualitatively and quantitatively for their nematode fauna. The average age of the orchards sampled, as nearly as could be determined, was 20 years. Each soil sample consisted of 1 qt. of orchard soil taken from around the feeder roots. During the first season the soil sample was taken from the root zone of a single tree selected at random in each orchard. Subsequently, as an aid to greater accuracy, a composite quart of soil was taken from the root zones

of four trees selected at random in the remaining 70% of the orchards. However, the data obtained by the two sampling methods were found later to be essentially similar. Each soil sample was processed by a modification of Cobb's sieving and decanting procedure by using screens of 35, 100, 200, and 325 mesh. The nematodes removed were identified and counted, and the populations standardized on the basis of 1 lb. of soil. Whenever possible, the following information was obtained at the time of sampling: general condition of the orchard, age of the orchard, soil type, drainage, type of cover crop, and history of replant difficulties.

Fig. 1 shows that only a few parasitic forms were found to be abundant in Ontario peach orchards.

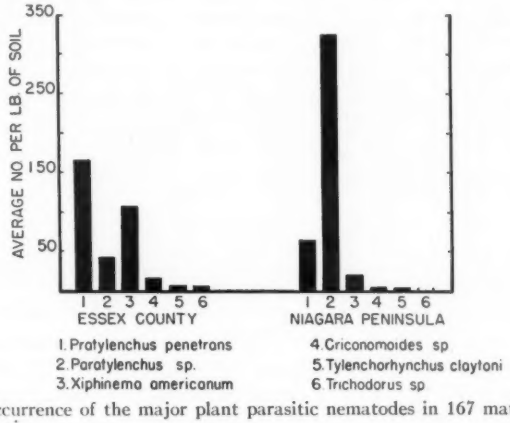

FIG. 1. Occurrence of the major plant parasitic nematodes in 167 mature peach orchards of Ontario.

The most frequently encountered plant parasitic forms were as follows:

Pratylenchus penetrans (Cobb, 1917) Sher & Allen, 1953. Over 99% of *Pratylenchus* identified were of *P. penetrans*. But occasionally *Pratylenchus minyus* Sher & Allen, 1953, occurred.

Paratylenchus sp. Those specimens which have been examined resemble most closely *Paratylenchus projectus* Jenkins, 1956. Other species may occur but, as no study was made of the relationship of this genus to the growth of peach, and in view of the present confused status of the taxonomy of this genus, the authors did not attempt to make specific identifications.

Xiphinema americanum Cobb, 1913.

Criconomoides rusticum (Micoletzky, 1915) Taylor, 1936. A number of specimens from several collections proved to be this species. However, Brown (1) has identified *Criconomoides xenoplax* Raski, 1952, from peach soil at Harrow, and other species may occur.

Tylenchorhynchus claytoni Steiner, 1937.

Trichodorus sp. is not a common nematode in southwestern Ontario and occurred in few orchards.

Species of *Longidorus* and *Rotylenchus* were recovered rarely. As is common in many soils, numerous other spear-bearing forms were encountered. These included many *Dorylaimina*, several species of *Aphelenchoides*, *Aphelenchus avenae*, *Tylenchus filiformis*, other species of *Tylenchus* and *Ditylenchus intermedius*. A list of over 25 genera which have been found in peach soils could be given but such a list would be of questionable value in a work of this nature. The occurrence of many forms could not be correlated with the disease. Most of them are quite common in Essex County, and occur in the same general soil types near the roots of many different crops.

Further reference to Fig. 1 shows that only three parasitic forms are common in peach soils. Relatively large populations of *Pratylenchus penetrans* and *Xiphinema americanum* occur in Essex County, whereas the predominant form in the Niagara Peninsula is *Paratylenchus* sp. Attempts to correlate populations of the three forms with a past history of replant difficulties in each of the orchards sampled indicated that neither *Xiphinema americanum* nor *Paratylenchus* sp. were consistently associated with histories of replant failure, but populations of *Pratylenchus penetrans* were positively correlated with the replant problem and this correlation is shown in Table I.

TABLE I

THE RELATION OF THE DISTRIBUTION OF *Pratylenchus penetrans* TO THE OCCURRENCE OF THE PEACH REPLANT PROBLEM IN THE TWO MAJOR ORCHARD AREAS IN ONTARIO

District	Number of orchards sampled	Per cent of total orchards sampled	Per cent of orchards with a replant history	*Pratylenchus penetrans* per pound of soil	
				Orchards with replant history	Orchards without replant history
Essex County	28	8.2	50.0	213	69
Niagara Peninsula	139	7.7	8.6	200	53

Examination of the data presented in this section shows the following:

(1) The proportion of orchards having a history of replant difficulties was greater in Essex County than in the Niagara Peninsula.

(2) *P. penetrans* was present in large numbers in proportionately more orchards in Essex County than in the Niagara Peninsula. In Essex County, 50% of the orchards sampled contained an average of 200 *P. penetrans* per pound of soil, whereas scarcely 9% of the Niagara orchards had populations as large.

(3) Regardless of the district, there were between three and four times as many root lesion nematodes (*P. penetrans*) in the soil of those orchards having a definite history of replant difficulties as in the soil of orchards where no replant difficulty has occurred.

The Occurrence of the Peach Replant Problem in Relation to the Limiting Effect of Certain Soil Types on *Pratylenchus penetrans*

The authors have established that high populations of *P. penetrans* occur in a greater percentage of orchards in Essex County than in the Niagara Peninsula, despite the similarity in climate and cultural practices. It was thought that differences in the physical characteristics of the soils, as observed during the soil screening process, might be responsible for limiting the numbers of the nematode. In Essex County, many peach orchards are located either on Harrow sandy loam or Fox sandy loam, both of which are relatively coarse soils according to the soil survey of Richards *et al.* (6). In contrast, according to Dr. J. A. Archibald (private communication), well over 80% of the peach soils in the Niagara Peninsula are classified either as Vineland fine sandy loam or Vineland sandy loam, both of which are relatively fine soils.

Sleeth and Reynolds (7) showed that infestation of *Sesbania exaltata* by the root-knot nematode *Meloidogyne javanica* was extremely light when the plant was grown on a fine-textured or clay-loam soil and very heavy in a coarse-textured or loamy-sand soil. Unpublished information by Mountain has shown that corn growing in Harrow sandy loam infested with *Pratylenchus minyus* supported a higher root population than did corn growing in Tuscola fine sandy loam or Brookston clay. In New York State Parker and Mai (4) found that in sour cherry orchards poor growth associated with *P. penetrans* was most evident in light-textured soils.

To determine whether numbers of *P. penetrans* were related to general soil types in Ontario peach orchards, soil samples from all commercial peach nurseries were evaluated for such differences. After the soils had been screened and the populations of nematodes determined, the average population of *P. penetrans* per pound of soil in each of the general soil types was calculated. The data are given in Fig. 2, which shows that the coarse soils contained larger populations of *P. penetrans* than the finer soils which are composed of higher

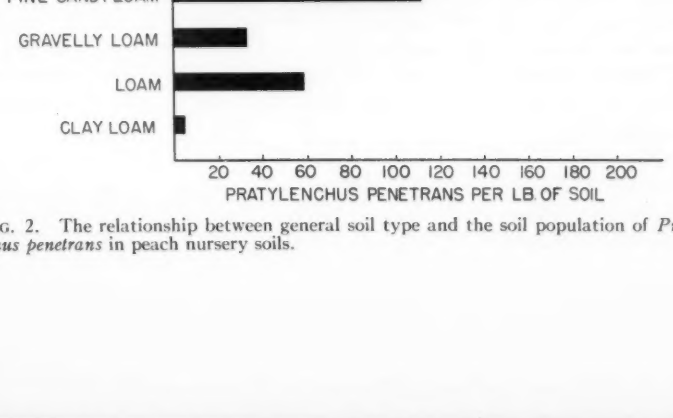

FIG. 2. The relationship between general soil type and the soil population of *Pratylenchus penetrans* in peach nursery soils.

proportions of silt and clay. It appears logical to assume that the lower populations of *P. penetrans* in peach soils of the Niagara Peninsula may be due to higher proportions of finer soil particles.

An experiment was undertaken in the greenhouse to test the assumption developed in the preceding paragraph. Quantities of soil were obtained from a peach orchard at the Vineland Horticultural Experiment Station at depths ranging from 6 to 18 inches. The soil, which is classified as Vineland fine sandy loam, may be regarded as representative of a typical Niagara peach soil and will be referred to in this section as Niagara Soil. By the same method, quantities of soil, classified as Harrow sandy loam and representative of Essex County peach soil, were obtained in the Harrow district and will be referred to in this section as Harrow Soil.

The soils were treated with methyl bromide at a rate of 2 lb. per 75 cu. ft. and then aerated for 1 month at the end of which period living nematodes could not be detected in either soil. Both soils were then inoculated with *P. penetrans*, the inoculum being prepared as follows: Vetch was planted in peach soil for a period of 1 month. At the end of that time, the vetch roots were heavily infested with *P. penetrans*. The roots were removed from the soil, washed, chopped, and added to steamed compost soil. Vetch was then planted in this soil. The nematodes readily penetrated the new vetch roots, and when representative samples of these roots were processed it was found that 98.2% of the nematodes that emerged from them were *P. penetrans*. The remainder were common saprophytes, mainly species of *Rhabditis* and *Eucephalobus*. During an incubation period of 1 month, an average of 2860 *P. penetrans* emerged per gram (fresh weight) of root.

In January 1957, the infested vetch roots were removed from the compost soil, washed, chopped, and mixed. One gram of the root material was mixed with sufficient fumigated Niagara Soil to fill one 7-in. pot which had been steamed previously. Twenty such pots were set up for both the Niagara and Harrow soils with corresponding checks. One Lovell peach seedling (approximately 4 cm. high) was planted in each pot. The initial height of each seedling was recorded. The pots were placed on a greenhouse bench in a random design.

The growth of the seedlings was measured at weekly intervals over a period of 6 weeks. The average net growth for each treatment, plotted at weekly intervals, is shown in Fig. 3. At the end of the experiment, the plants were removed from the soil, the roots washed thoroughly and incubated (10) for a period of 1 month. The only parasitic nematode to emerge from the roots was *P. penetrans* and the populations of this nematode were standardized on the basis of numbers per gram (dry weight) of root tissue. The results are given in Table II.

Samples of the two soils were sent to the Department of Soils at the Ontario Agricultural College for particle size analysis. Later, it became apparent that quite large differences occurred in the sand fraction of the soils and further analyses were carried out. The particle size analyses are recorded in Table III.

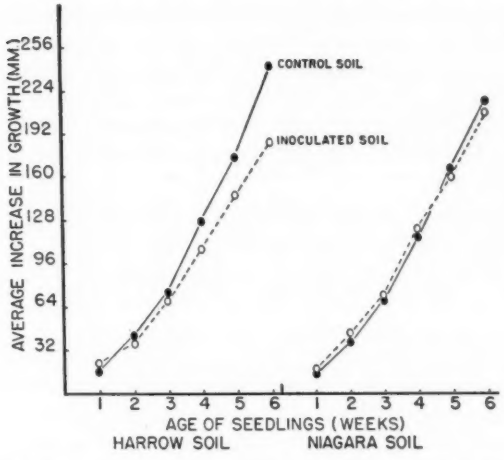

FIG. 3. Comparison of the growth of peach seedlings in nematode-inoculated and in noninoculated soils of the two major peach-producing areas of Ontario.

TABLE II

RELATIVE GROWTH OF PEACH SEEDLINGS AND FINAL POPULATIONS OF *Pratylenchus penetrans* IN ARTIFICIALLY INFESTED SOILS TYPICAL OF ESSEX COUNTY AND THE NIAGARA PENINSULA ORCHARDS

Soil type	Growth of peach seedlings (as % of controls)	*Pratylenchus penetrans*	
		Original inoculum (per pot)	Final recovery (per gram of root)
Harrow Soil	77%	2860	2258
Niagara Soil	97%	2860	877

TABLE III

PARTICLE SIZE OF PEACH SOILS TYPICAL OF ESSEX COUNTY AND THE NIAGARA PENINSULA

Soil	Distribution of soil particles (%)					
	Sand				Silt	Clay
	2.0–0.42 (mm.)	0.42–0.144 (mm.)	0.144–0.044 (mm.)	0.044–0.02 (mm.)	0.02–0.002 (mm.)	Less than 0.002 (mm.)
Harrow	7	58	14	1	7	13
Niagara	1	10	51	12	11	15

The data presented in Tables II and III and Fig. 3 support the following conclusions:

(1) The growth of peach seedlings in nematode-infested Harrow Soil was significantly suppressed by the end of the third week, and the suppression subsequently increased for the duration of the experiment. Only a slight and insignificant suppression of growth occurred in the seedlings in the nematode-infested Niagara Soil.

(2) There were no significant differences between growth of seedlings in the noninfested Niagara Soil and noninfested Harrow Soil.

(3) Although the two soils initially were inoculated with identical numbers of *P. penetrans*, at the conclusion of the experiment scarcely one-third as many nematodes were recovered per gram of peach root grown in Niagara Soil as from those grown in Harrow Soil. There is no doubt that some factor in the Niagara Soil had limited the numbers of *P. penetrans* within the roots.

(4) Physical analyses of the two soils have shown that the sand fraction of the Harrow Soil is much coarser than that of the Niagara Soil. Indeed, 63% of the Harrow Soil consists of particles larger than 0.15 mm. as compared with 11% of the Niagara Soil.

If it be assumed that *Pratylenchus penetrans* is an incitant of the peach replant problem, an explanation for the regional differences in severity of the problem becomes relatively simple. The populations of *P. penetrans* appear to have been limited by the soils of the Niagara area and therefore the nematode has seldom built up to the point where noticeable symptoms on replants would appear.

It is also interesting to recall that not all of the replanted orchards in Essex County have shown symptoms of replant failure. Occasionally the disease is severe in only a portion of a newly replanted orchard. A possible explanation for the variation of the problem in Essex County may be found in studying a soil map of the area. The soils of the peach-growing areas of this county are quite variable. Richards *et al.* (6) have noted that these soils were subjected to strong morainic influences resulting in small stoney ridges occurring among outwash soils giving quite sharp fluctuations in soil type within relatively small areas. In one orchard the authors observed that the soils varied from coarse sand to sandy loam and that replant failure was more severe in the coarse soil. From the data presented, one might predict that the peach replant problem is likely to be most severe on the coarser sands, and somewhat less severe or perhaps even nonexistent on fine sandy loams or silty loams.

Discussion

The rules of proof of pathogenicity which were developed in 1882 by Robert Koch (3) gave bacteriologists a sound and logical procedure for etiological investigations of diseases of man and animals. The fact that the same rules have proved so valuable to plant pathologists in their investigations attest to the sound logic and importance of these postulates.

In the study of root-rot complexes, the word complex suggests an intricate problem because of the possible interaction of many factors in the onset of the disease. The first of Koch's postulates is that there must be a close and consistent association of the organism with the disease, therefore the application of this first postulate should resolve the relative importance of the various potential factors in the etiology of a root rot. Laboratory procedures may demonstrate a number of reactions which can cause root degeneration, but if a close association between such a reaction and the disease, as it actually occurs in nature, has not been shown, the basis of this reaction in terms of reality can never be established. The mechanism or organism remains a potential or so-called contributing factor and the problem continues to appear vague and complex.

The authors believe the only way that the peach replant problem or any root rot can be clarified is by concentrating on those factors which are closely associated with the disease. Early in this investigation, the authors found some 25 genera of nematodes in peach soils. All of these were potential factors in the peach replant problem and the literature shows that many of them do affect the growth of plants. Initially, then, the nematode picture appeared to be very complex. But the application of Koch's first rule of proof indicated that only *P. penetrans* should be a potential cause of the failure of replanted peach trees. The authors are convinced that the data are adequate to postulate that the close association between the distribution of the peach replant problem and high populations of *P. penetrans* would seem to justify further investigation of the host–parasite relations involved.

Acknowledgments

The authors extend sincere appreciation to staff members of the Provincial Horticultural Experiment Station, Vineland Station, Ontario, who gave valuable assistance. Dr. J. F. Brown, formerly research scientist at Vineland, was of great help with many of the earlier phases of the work in the Niagara Peninsula. Dr. J. A. Archibald, research scientist at Vineland, supervised the collection of most of the soil samples required for the nematode surveys. He also provided the authors with information on soils of the Niagara area.

In the very early phases of this work, the identification of the various nematodes encountered was confirmed by Mr. Gerald Thorne, formerly Senior Nematologist, U.S. Department of Agriculture.

The authors would like to acknowledge the assistance of Professors N. R. Richards and L. R. Webber of the Soils Department, Ontario Agricultural College, for conducting soil analyses.

Finally, the authors would like to thank Mr. E. J. Murray, technician, who helped with the screening and handling of the hundreds of soil samples processed during this study.

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THE PEACH REPLANT PROBLEM IN ONTARIO

VI. THE RELATION OF PRATYLENCHUS PENETRANS TO THE GROWTH OF YOUNG PEACH TREES[1]

W. B. MOUNTAIN AND H. R. BOYCE[2]

Abstract

Further evidence was accumulated indicating that the peach replant disease decreases in severity as the interval increases in time between removal of the old orchard and the planting of new trees. The ameliorating effect of the interval may be related to the decline of *Pratylenchus penetrans* (Cobb, 1917) Sher & Allen, 1953. In experiments, increased growth of peach was associated with the control of *P. penetrans* by nematocides and *P. penetrans* was found to be the first nematode to attack newly developing peach roots. The populations of *P. penetrans* within such roots increased rapidly, then gradually declined. Generally, ectoparasites appeared later, increased relatively slowly, and occurred infrequently until late in the second growing season. The soils of all commercial peach nurseries in Ontario contain *P. penetrans* and the nematode may be transported in the roots of nursery stock.

Introduction

In another paper Mountain and Boyce (3) showed that regional differences in the severity of peach replant failure were related to the relative numbers of *P. penetrans* present in the soils. Accordingly, the relation of the nematode to the difficulty of obtaining satisfactory stands and growth of replanted peach trees was investigated by further field studies and experimentation by the use of nematocides which might also result in developing a satisfactory method of control.

Severity of the Problem and Numbers of *Pratylenchus penetrans* in Relation to the Replanting Interval

Early observations indicated that the severity of the problem increased with decreasing intervals between removal of the old trees and planting young ones. The most severe symptoms usually appeared when mature trees were removed in the fall and the land was replanted to peach the following spring. Frequently, when this interval was 3 or more years the problem was not apparent. However, further observations showed that in several of the most serious replant problem orchards in Essex County trouble was experienced after the old trees had been removed for 6 or more years. Variation in severity of the problem in relation to time may be due to the causal organism or organisms being maintained on other plants in the absence of peach. If suitable host crops are grown on the land, the pathogens may remain at a high level in the soil for several years. In the absence of such hosts, the pathogens are likely to decline rapidly and the disease might disappear in a relatively short time.

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Joint contribution from the Botany and Plant Pathology Division (No. 1645) and the Entomology Division (No. 3703), Science Service, Canada Department of Agriculture, Ottawa, Ontario.

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In one orchard where experiments were conducted, some of the mature trees were removed in the fall of 1951 and the remainder in the fall of 1954. In the spring of 1955, the entire block was replanted to peach. In the years between 1951 and 1955, the portion from which the trees had been removed longer was planted to a variety of vegetable crops including cucurbits.

By late summer of 1955, it was obvious that the young peach trees were in much better condition in that portion of the orchard where the trees had been removed for 3 years than where the trees had only been removed the previous fall. In October 1955, 20 trees were selected at random from each portion of the orchard, the growth of each tree for the season was measured and the nematode populations were determined by soil screening. These data are presented in Table I.

TABLE I

GROWTH OF YOUNG PEACH TREES AND POPULATIONS OF PLANT PARASITIC NEMATODES IN RELATION TO THE INTERVAL BETWEEN REMOVAL OF THE PRECEDING ORCHARD AND REPLANTING

Interval between removal of mature orchard and replanting, months	Growth of peach replants in 1955 (planted in May, 1955)		Plant parasitic nematodes per lb. of soil	
	Linear growth, cm.	New branches produced	*Pratylenchus penetrans*	Others
44	609	23	9	5
8	275	13	180	53

It might be reasoned that the crops planted in the years between 1951 and 1955 in that portion of the orchard where the mature trees had been removed for the longer period were unsuitable hosts for *P. penetrans* and therefore the population of this parasite had declined. In contrast, in that portion of the orchard which was replanted after the shorter interval, the population of *P. penetrans* would have declined only slightly, especially as winter conditions prevailed for most of the time that the nematode was without a suitable host. Consequently, when the entire block was replanted to peach, one portion of the replanted area was exposed to a much higher inoculum level than the other. The difference in growth, as shown in Table I, reflects the difference in inoculum levels.

Experiments

Modification of Growth of Peach Replants and Populations of Pratylenchus penetrans by the Use of Nematocides

In Several Orchards Using Lovell Peach Seedlings

In the spring of 1954, an experiment was designed for the study of effects of certain chemical soil treatments upon soil microflora, nematodes, and root

aphids in relation to the growth of peach seedlings. Some of the data from this experiment have already been reported (6) and the present paper deals only with the nematode portion of the experiment.

Six peach orchards were selected, from which the mature trees had been removed during the autumn of 1953. In each orchard, plots, each of 125 sq. ft., were located in the intersite areas of the preceding orchard so as to avoid any possible site effects from toxins (Patrick (4)). Randomized soil treatments were applied to these plots. The treatments were as follows: (1) ethylene dibromide (Dowfume W-40) applied by hand injector at 40 Imperial gallons per acre; (2) dichloropropane–dichloropropene mixture (Shell D-D) applied by hand injector at 40 Imperial gallons per acre; (3) chlorobromopropene (Shell CBP-55) applied as a soil drench in split application to give a total rate of 33 Imperial gallons per acre; (4) methyl bromide (Dowfume MC-2) applied under gas-tight covers at 2 lb. per 125 sq. ft. Untreated plots served as controls.

Each plot was planted to 15 Lovell peach seedlings in three rows 14 days after the final soil treatment. The height of each plant was measured.

In October, a soil sample from each plot was screened for nematodes and the populations of parasitic nematodes were calculated per pound of soil. In each orchard the predominant plant parasitic nematode was *P. penetrans*. The numbers of this nematode and the total net linear growth of the trees in each plot are given in Table II.

TABLE II

GROWTH OF PEACH SEEDLINGS AND POPULATIONS OF *Pratylenchus penetrans* AS AFFECTED BY SEVERAL CHEMICAL TREATMENTS OF THE SOIL

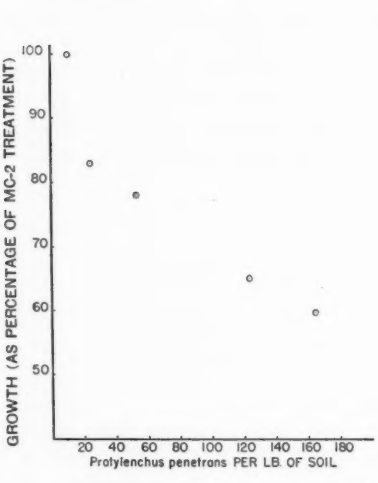
Soil treatment	Average net increase in growth, in.	Average population of *P. penetrans* per lb. of soil
Methyl bromide	1749	11
Dichloropropane–propene	1456	25
Ethylene dibromide	1363	54
Chlorobromopropene	1130	124
Check	1029	165
LSD .05	407	75

Table II shows that there was a measurable increase in growth as a result of soil fumigation. However only the treatments D-D and MC-2 resulted in a significant growth response. There is little doubt that growth in the plots treated with MC-2 was superior to that in the plots treated with D-D, but in this experiment such growth improvement was not statistically significant. There is an indication that MC-2 was somewhat superior to D-D in its nematocidal effect.

Further examination of the data in Table II suggested that growth of the peach seedlings may be related to the population of *Pratylenchus penetrans*. In Fig. 1 growth (as percentage of that in the plots treated with MC-2) is

plotted against the nematode population. With the LSD values of Table II in mind, a cautious interpretation of Fig. 1 suggests that peach growth declines with increasing populations of *P. penetrans*.

There was great variability of growth in the orchards. For example, the average growth increase in the six plots treated with MC-2 was 1749 in. but the standard deviation was 559 in. The variation in growth represents the effect of factors other than those which might be controlled by MC-2. Obviously such factors would include variation in soil type and condition, varying nutrient levels and soil moisture, as well as the heterogeneity of the Lovell seedlings. However, as Wensley (6) has noted, the relative effectiveness of the fumigants did not vary greatly from orchard to orchard. Accordingly, the authors believed that variability of data could be reduced by conducting an experiment in a single orchard and by increasing the number of replications.



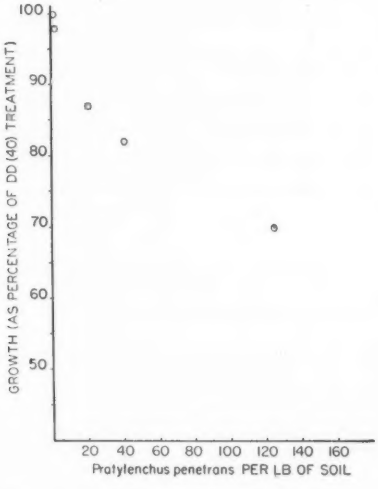


FIG. 1. Apparent relation between growth of peach seedlings (expressed as a percentage of the growth in plots treated with MC-2) and population of *P. penetrans* in the soil.

FIG. 2. Apparent relation of growth of peach nursery stock, as percentage of the growth in the plots treated with D-D at 40 gal. per acre, to the soil population of *P. penetrans*.

In Single Orchards Using Commercial Nurserly Stock

It was decided that the variability of growth might be reduced by: (1) conducting experiments in single orchards where there had been definite evidence of a replant problem, in order to reduce the variation in the environment; (2) making the number of replications as large as practicable; (3) replacing peach seedlings by commercial nursery stock. The last step would simulate more closely the conditions under which the problem develops in

the field. Also, it was thought that the use of nursery stock budded with a single variety would reduce the heterogeneity of growth to some extent, since the aboveground part of the plant is propagated vegetatively.

(a) Effectiveness of Preplanting Nematocidal Treatments (Orchard A)

An orchard in the Harrow area, in which the problem had been particularly severe, appeared suitable. The mature orchard had been removed in the fall of 1949, and was replanted in the spring of 1950. By the spring of 1951 mortality had been so great that the entire block was removed and the ground planted to potatoes and sweet potatoes in 1951 and 1952. In the spring of 1953 a second attempt was made by the owner to re-establish the peach orchard. By the spring of 1954, approximately 15% of the young trees had died and during the summer an additional 30% either died or grew poorly. All dead and moribund trees were removed in September 1954.

In October 1954 a soil fumigation experiment in randomized block design was undertaken in this orchard. Plots, each 10 ft. by 10 ft., were laid out in a linear manner to conform with the tree rows in the orchard. The center of each plot conformed exactly with the former site of a dead replant. The soil treatments applied by hand injector to a depth of 6 in. on 10-in. centers were as follows: (1) dichloropropane-dichloropropene mixture (Shell D-D) at 40 Imperial gallons per acre; (2) Shell D-D at 30 Imperial gallons per acre; (3) ethylene dibromide (Dowfume W-40) at 40 Imperial gallons per acre; (4) Dowfume W-40 applied at 30 Imperial gallons per acre. In the check no chemical was added to the soil. Each treatment was replicated 15 times.

In May 1955 commercial nursery stock of the variety Golden Jubilee was selected. The roots of these trees were dipped in hot water at 114° F. for 10 minutes in an attempt to kill any nematodes that might be within the roots. One tree was then planted in the center of each plot. Subsequent greenhouse experiments with a portion of the nursery stock indicated that the material was not infested with nematodes. The circumference of the trunk, the height, and number of branches were recorded for each tree at time of planting. Since differences in growth were the same regardless of the method of measurement only the data for trunk circumference are used in this paper.

In October 1955 soil samples from all treatments were obtained and screened to remove the nematodes. The population of plant parasitic nematodes was determined for numbers and kind in each case and equated on the basis of numbers per pound of soil. Almost all of the parasites recovered were *P. penetrans*. At the same time the net growth for each tree was determined by measuring the circumference at a distance 9 in. above the soil surface. An analysis of variance was applied to the data using a square root transformation (5). The data are recorded in Table III.

An examination of Table III shows that a significant increase in growth occurred in the plots treated with ethylene dibromide. However, the growth of plants in soil treated with D-D appeared to have been suppressed, especially at the higher dosage. There is every indication that the suppression of growth was due to phytotoxicity. The color of the foliage was abnormally dark

green, the twigs were thicker and had a marked reddish cast, and internodes were shorter. The fruit buds were abnormally large and more abundant than those of plants grown in the other treated plots. However, as will be shown later, the phytotoxic effects lasted for the first year only.

It is also apparent from Table III that all treatments markedly reduced the soil populations of *P. penetrans* and that this nematode was completely absent from the D-D (40) plots.

In October 1956, soil samples again were obtained from each plot and screened to remove the nematodes. Also the increase in growth was determined as before. It was noticed at this time that several trees in the check plots had died whereas others were affected seriously with basal cankers and had been attacked by the lesser peach tree borer, *Synanthedon pictipes* (G. & R.), as shown in Table IV. The data for growth of the living trees in 1956 and the soil populations of *P. penetrans* are given in Table V.

TABLE III

THE GROWTH OF YOUNG PEACH TREES AT THE END OF THE FIRST GROWING SEASON IN RELATION TO SOIL TREATMENT AND NUMBERS OF *Pratylenchus penetrans*

Soil treatment	Net increase in circumference, cm.	Average number of *P. penetrans* per pound of soil	
		Actual	$\sqrt{x+0.5}$ transformation
Check	0.97	234	15.15
EDB (30)*	1.51	18	4.17
EDB (40)†	1.62	7	2.65
DD (30)‡	1.45	1	1.00
DD (40)§	0.89	0	0.71
LSD .05	0.36	—	1.89

*,† Ethylene dibromide at 30 and 40 Imperial gallons per acre respectively.
‡,§ Dichloropropane–dichloropropene mixture at 30 and 40 Imperial gallons per acre respectively.

TABLE IV

CONDITION OF YOUNG PEACH TREES AFTER THE SECOND GROWING SEASON IN RELATION TO SOIL TREATMENT

Soil treatment	Trees affected, %			
	Dead	Basal canker	Lesser peach borer	Total
Check	20	7	13	40
EDB (30)	7	0	0	7
EDB (40)	0	7	0	7
DD (30)	0	0	7	7
DD (40)	0	0	0	0

TABLE V

THE GROWTH OF YOUNG PEACH TREES AT THE END OF THE SECOND GROWING SEASON IN RELATION TO SOIL TREATMENT AND NUMBERS OF *Pratylenchus penetrans*

Soil treatment	Net increase in circumference, cm.	Average number of *P. penetrans* per lb. of soil	
		Actual	$\sqrt{x+0.5}$ transformation
Check	5.02	125	11.11
EDB (30)	5.90	49	6.92
EDB (40)	6.20	21	4.43
DD (30)	7.03	2	1.51
DD (40)	7.16	1	0.94
LSD .05	—*		1.64

*Analysis of variance could not be applied because of missing data (C. Reimer, private communication).

It is apparent from Table IV that for at least 2 years practical control of peach replant failure was obtained. In the treatment which was most effective against nematodes there was no mortality, no sign of canker or borer activity. This indicates the very healthy condition of the trees growing in soil treated with D-D at 40 gal. per acre.

As indicated by Table V, in the second year the phytotoxic effects of D-D disappeared and seasonal growth was greater in the D-D treated soils. The fumigant D-D continued to give outstanding control of *P. penetrans* and it would appear that, when soil fumigation is effective, repopulation of the soil by this nematode is very slow.

There was apparently a similar relation between peach growth and the numbers of *Pratylenchus penetrans* in the soil as was found in the preceding experiment. In Fig. 2, growth, as a percentage of that in the D-D (40) treatment, plotted against the nematode population, shows the relationship.

From a comparison of Fig. 2 with Fig. 1 it was found that the values of the latter could be superimposed upon Fig. 2 and the two sets of points then fitted a single line. This suggests that, regardless of the location or whether growth is measured as linear extension or trunk circumference, differing populations of *P. penetrans* are associated with differential effects upon growth. This relationship suggests that growth response of peach may be correlated with the numbers of *P. penetrans* in the soil.

The variability of the growth data, although greatly reduced by the techniques used in this experiment, was not eliminated and differences in growth between the blocks again were statistically significant. A possible reason for the remaining variability might be the distribution of *P. penetrans* in the soil of this orchard. Accordingly, a root sample was collected from every tree in the experiment to measure further the variability of the distribution

of *P. penetrans*. The samples were incubated (7) for 2 months, then the populations of *P. penetrans* that had emerged from the roots were counted and standardized on the basis of numbers per gram of dry root. The average population and the standard deviation were calculated for each of the treatments and the results are summarized in Table VI.

It might be argued that the large standard deviations of the numbers of *P. penetrans* in relation to treatment represent variability in nematocidal effectiveness, but the size of the standard deviation for the check suggests rather that the distribution of the nematode was extremely erratic.

In the orchard in which the experiment was conducted the soil type varied from coarse to fine sand. Mountain and Boyce (3) showed that the population of *P. penetrans* varies in relation to size of soil particles. Therefore differences in the physical composition of the soil may account for the uneven distribution of the nematode in the experimental plots.

TABLE VI

VARIABILITY IN EXPERIMENTAL PLOTS OF *Pratylenchus penetrans*

Soil treatment	Av. population per g. of root	Standard deviation
Check	222	424
EDB (30)	127	181
EDB (40)	59	53
DD (30)	68	107
DD (40)	9	9

(b) Effectiveness of Postplanting Nematocidal Treatments (Orchard B)

In 1954, McBeth (2) reported that an experimental Shell product known at that time as OS 1897 appeared to be approximately 10 times more effective nematocidally than D-D and was relatively nontoxic to many plants. If this chemical were relatively nonphytotoxic to peach and had nematocidal values equal to those of other fumigants presently available, it might be of great value as an immediate preplanting or postplanting treatment in reducing the difficulty of re-establishing peach trees.

The OS 1897 contained approximately 97% of the halogenated hydrocarbon 1,2-dibromo-3-chloropropane. This product is now sold under the trademark Nemagon by the Shell Chemical Company. A similar formulation, Fumazone, containing approximately 35% of the halogenated hydrocarbon and marketed by the Dow Chemical Company, was also used in this test. Accordingly, the purpose of this experiment was to test formulations of 1,2-dibromo-3-chloropropane with regard to phytotoxicity and control of plant parasitic nematodes, and to determine whether the growth of peach replants could be improved.

A test of the phytotoxicity of Nemagon to peach was conducted in the greenhouse during the winter of 1954–1955, before the field experiment was started. Peach seedlings were planted in steamed soil in 5-in. pots and various concentrations of Nemagon were added to these pots as emulsified drenches as shown

in Table VII. A random block design was used and there were 20 replicates of each treatment. The net increases in linear growth of the seedlings at the end of 5 weeks are given in Table VII.

It is apparent from Table VII that, under the conditions of this experiment, Nemagon was not phytotoxic to peach seedlings at concentrations of 1.5 to 15 U.S. gallons per acre over a period of 5 weeks.

TABLE VII

THE EFFECT OF VARIOUS CONCENTRATIONS OF NEMAGON UPON THE GROWTH OF POTTED PEACH SEEDLINGS

Concentrations of Nemagon, U.S. gal. per ac.	Net increase in linear growth, cm.
0.0	15.7
1.5	15.0
3.0	16.6
5.0	13.7
15.0	16.1
30.0	4.6
40.0	5.1
LSD .05	6.0

Consequently an orchard in the Leamington area, from which the mature trees were removed in the fall of 1954, was selected for a field test of 1,2-dibromo-3-chloropropane. The peach replant problem had occurred previously in another section of this orchard. In May 1955, the grower replanted the block using commercial nursery stock of the variety Golden Jubilee.

Approximately four weeks later an experiment was set up to test the effectiveness of drenching the soil around the peach trees with 1,2-dibromo-3-chloropropane. The chemical was applied as an emulsified drench on the theoretical basis that, as the chemical diffused down into the soil, it would kill nematodes from the soil surface downwards. It was thought that this method of application might give better control of the nematodes near the soil surface than is obtained with soil injection. The materials used were as follows: (1) Nemagon at 10 Imperial gallons per acre; (2) Nemagon at 5 Imperial gallons per acre; (3) M-525 (Fumazone) at a rate corresponding to Nemagon at 5 Imperial gallons per acre. Untreated plots served as checks.

Plots, each 10 ft. by 10 ft., were laid out in rows so that one replant was situated in the center of each plot. A randomized block design of 20 replicates per treatment was used. After application, the chemical was washed into the soil with five Imperial gallons of water. The following day, 1 in. of water was applied to the entire block by irrigation. Ten days later, the size of each replant was determined by measuring the circumference in millimeters at a height of 9 in. above the soil surface.

In October 1955, soil samples obtained from all of the plots were screened for nematodes. Examination showed that, as in preceding experiments, most of the parasites recovered were *P. penetrans*. When the soil samples were

taken, the circumference of each tree was measured and the net growth increase for the summer of 1955 calculated. The data obtained are recorded in Table VIII.

Table VIII shows that the populations of *P. penetrans* were reduced, but were not effectively controlled by the chemical. Growth in the treated plots, although indicative, was not significantly better than growth in the check.

TABLE VIII

GROWTH OF YOUNG PEACH TREES AND POPULATIONS OF *Pratylenchus penetrans* AT THE END OF THE FIRST GROWING SEASON

Soil treatment Imp. gal. per ac.	Net increase in tree circumference, cm.	*P. penetrans* per lb. of soil
Check	0.96	200
Nemagon 5	1.10	133
Fumazone 14	1.15	135
Nemagon 10	1.14	44
LSD .05	N.S.	65

In relation to the data presented in Table VIII, the nematodes had a period of 1 month in which to penetrate and damage the roots before the chemical was applied and the initial root damage may have been sufficient to offset the subsequent beneficial effects of the chemical. Furthermore, information from the Shell Chemical Company indicated that 1,2-dibromo-3-chloropropane is relatively slow in its nematocidal activity. With these facts in mind, it was believed that the growth increase during the second year following fumigation might be more indicative of the true value of the chemical. Therefore, at the end of the second growing season, October 1956, soil samples were again obtained from each plot and screened for parasitic nematodes, and the net increase in growth was measured for each tree and the tree mortality in each treatment recorded. The information obtained is summarized in Table IX.

TABLE IX

MORTALITY AND GROWTH OF YOUNG PEACH TREES AND POPULATIONS OF *Pratylenchus penetrans* AT THE END OF THE SECOND GROWING SEASON

Soil treatment Imp. gal. per ac.	Tree mortality, %	Net increase in circumference, cm.	*P. penetrans* per lb. of soil
Check	10	4.16	168
Nemagon 5	15	5.20	56
Fumazone 14	10	4.77	58
Nemagon 10	10	5.12	60
LSD .05	—	N.S.	42

It is apparent from Table IX that 1,2-dibromo-3-chloropropane, as applied, was ineffective in improving growth although it reduced the numbers of *P. penetrans*. Mortality had not been reduced by the treatments and growth of the surviving trees was not significantly better than the checks. Apparently, the nematocidal effect of the chemical at the lower dosages continued for a considerable time after application, but effective control of the nematode was not obtained with any of the treatments. Possibly other methods of application, such as soil injection before replanting an orchard, might have given more effective control of *P. penetrans*.

Generic Components of Plant Parasitic Nematode Fauna in Fumigated and Nonfumigated Peach Soils and Changes with Time

During the investigation it became apparent that, as time passes, a consistent shift occurs in the parasitic nematode fauna associated with the roots of newly planted peach seedlings or nursery stock.

On the basis of many observations and preliminary field experiments, it is believed that *P. penetrans* is the first nematode to attack the roots of the newly planted seedling or nursery tree. Field observations have indicated that, within a very few days after the planting of peach seedlings, *P. penetrans* can be found within peach roots. In one instance, peach seedlings were planted in peach soil in the greenhouse. Three days later, the roots were removed from the soil, washed thoroughly, and placed in Baermann funnels. The only nematode to emerge from the roots was *P. penetrans*.

A second consistent observation is that during the first growing season the number of *P. penetrans* within the roots declines as the season progresses. Frequently the population increases again during the autumn of the first year; but it never reaches the level that occurred in early summer. During the second growing season the population decline of *P. penetrans* continues. In this connection the results of a preliminary experiment carried out with Dr. Z. A. Patrick during the summer of 1953 are of interest.

Peach seedlings were planted between the trees in a mature peach orchard and removed at intervals throughout the year. After being washed, the roots were placed in Baermann funnels for a week and the nematodes that emerged were identified, counted, and standardized on the basis of numbers per gram of root. The results of this experiment are summarized in Table X.

TABLE X

CHANGES IN POPULATION DENSITY OF *Pratylenchus penetrans* DURING THE FIRST GROWING SEASON OF PEACH SEEDLINGS

Date	Age of experiment, days	Average number of *P. penetrans* per g. of root
June 30	25	7507
July 7	32	6300
July 21	46	255
August 23	79	74
October 23	140	65
November 27	175	736

Unpublished research carried out by the senior author indicates that the optimum soil temperature for the build-up of *P. penetrans* is relatively low. Accordingly, supraoptimum soil temperatures may be one of the factors responsible for the rapid decrease of the nematode in midsummer. The tendency for the population to increase in the fall possibly results from the return of soil temperatures more conducive to the activities of this organism. However, it is obvious from Table X that the increase in the autumn was insufficient to return the population to the spring level.

In most of the peach orchards, in which observations were made, the ectoparasites did not occur in large numbers in the root zone of newly planted nursery trees or seedlings until midsummer. In one preliminary experiment ectoparasitic nematodes were not numerous around the roots of peach seedlings until August. However, the ectoparasitic populations increased 8-fold by mid-September and 20-fold by late November.

In some of the experiments recorded in the preceding section the shifts in abundance of parasitic forms were studied over a period of two growing seasons. Such data taken from the experiment conducted in Orchard A are shown in Fig. 3 and Fig. 4, and those from the experiment in Orchard B are shown in Fig. 5.

It is apparent from Fig. 3 that, by the end of the first growing season in this particular orchard, the generic complex in untreated areas was still quite simple. *Pratylenchus* was the predominant genus and only two ectoparasitic

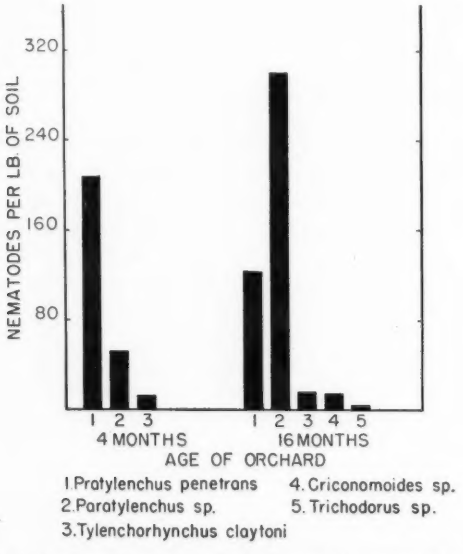


FIG. 3. Shifts in the generic components of the parasitic nematode fauna in untreated plots of Orchard A.

forms had appeared in relatively low numbers. By the end of the second growing season, the ectoparasites outnumbered *Pratylenchus*. The predominant form was *Paratylenchus* and two other ectoparasitic genera had appeared.

The generic shifts in the fumigated soils of Orchard A are also of interest (Fig. 4). At the end of the first growing season, practically no parasitic nematodes were present in plots that received either treatment. The populations of *Pratylenchus* in the soils treated with ethylene dibromide were somewhat higher than in the D-D treatment, as noted previously. The only ectoparasite present in either treatment was *Paratylenchus*. At the end of the second growing season there was an increase in *both* the endoparasite and the ectoparasites, although the latter predominated. The authors believe that the increase of *Pratylenchus* during the second season is an indication of the presence of a substrate of succulent, healthy root tissue suitable for its build-up.

It is evident from Fig. 5 that there was a similar trend in Orchard B from a relatively simple generic complex consisting largely of the endoparasite *Pratylenchus* to a more involved complex of both the endoparasite and ectoparasites. In this orchard, the decline in the population of *Pratylenchus* was not so marked. The population of all of the ectoparasitic forms increased during the second season and two additional genera appeared.

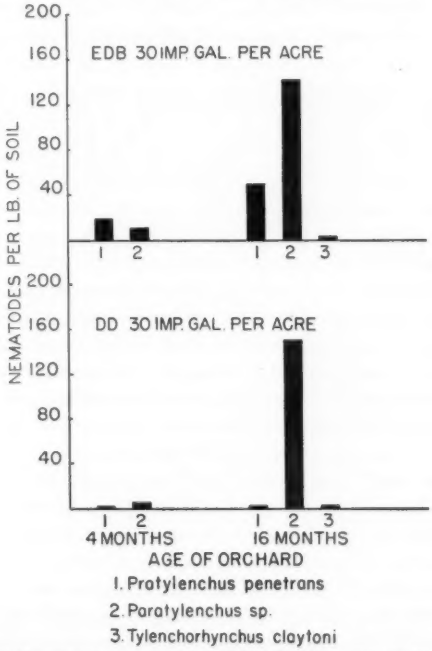


FIG. 4. Shifts in the generic components of the parasitic nematode fauna in fumigated plots of Orchard A.

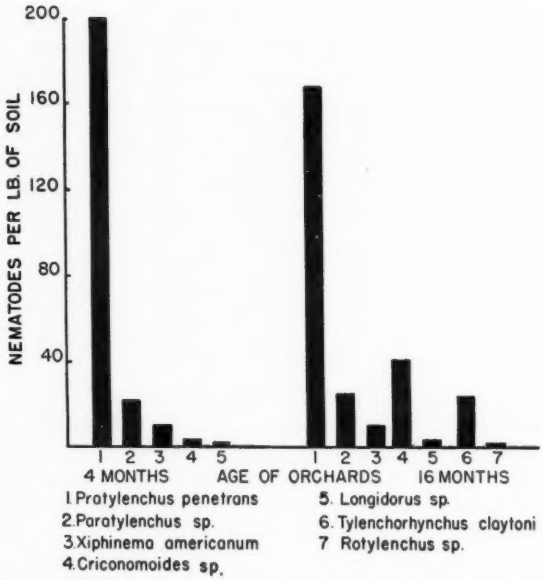


FIG. 5. Shifts in the generic components of the parasitic nematode fauna in untreated plots of Orchard B.

Parasitic Nematode Populations of Commercial Peach Nursery Soils

In a previous section of this paper, the authors demonstrated the beneficial effects to peach trees that were planted in old orchard soils after nematocidal soil treatments. Such benefits may only occur and be maintained if parasitic nematodes are not reintroduced into the fumigated soil. It was considered that the roots of nursery stock may be infested with parasitic nematodes, and the soil may be reinoculated from this source.

A soil survey of all major commercial peach nurseries in Ontario to establish the distribution of plant parasitic nematodes in such locations was completed in 1956. In each nursery a composite soil sample was taken from the root zone of four young trees selected at random. Each soil sample was screened in the usual manner and the nematodes were identified and counted, and the populations standardized on the basis of numbers per pound of soil. The results of the survey are summarized in Table XI.

Table XI shows that all of the major peach nurseries in the province were infested with *P. penetrans* and that no other endoparasite was present. Peach roots, collected from the nurseries at the same time as the soil, were placed in Baermann funnels for 1 week and only *P. penetrans* was found.

Although the authors recognized the possibility of the ectoparasites being transported with the nursery stock in soil particles adhering to the roots, they were more concerned with the possibility of *P. penetrans* being carried within the living roots.

TABLE XI

PARASITIC NEMATODES IN THE SOIL OF COMMERCIAL PEACH NURSERIES OF ONTARIO

Genera	Number per lb. of soil*	% of nurseries infested
Pratylenchus†	98	100
Paratylenchus	42	90
Tylenchorhynchus	10	26
Xiphinema	10	16
Trichodorus	12	11

†Only *P. penetrans* was found.
*Average population of nurseries infested with the genus.

Examination of the root system of a typical nursery tree showed that most of the finer root system had been lost as a result of digging, subsequent handling, and pruning of injured roots. Accordingly, almost all of the roots which remain are fairly large and quite heavily suberized. The question arises as to whether an endoparasitic nematode such as *P. penetrans* will parasitize such roots and emerge as viable inoculum when the nursery tree is planted. In the fall of 1956, root samples were collected in the Science Service orchard from 10 young peach trees of the same age as nursery trees dug for shipment. All of the smaller roots under 1/16th in. in diameter from each sample were carefully removed and incubated. The larger, suberized roots were then incubated separately. At the end of 1 month an average of 1140 *P. penetrans* had emerged from the samples of root under 1/16th in. in diameter and 36 from the samples of larger roots. Thus, a few *P. penetrans* penetrate larger peach roots and are capable of emerging from them.

Discussion

The results of field investigations presented in this paper support and strengthen the conclusion developed in the preceding paper that there is a close and constant association between the peach replant problem in Ontario and the root lesion nematode *Pratylenchus penetrans* in terms of distribution, occurrence, and severity of the disease.

The fact that the greatest growth of replanted trees resulted from use of the most effective nematocide suggests that nematodes are of considerable importance in the failure of peach replants.

The experiments with nematocides, as far as they have progressed, indicate that the problem of establishing peach on former orchard locations can be reduced by the use of several such materials. Although methyl bromide appears to have somewhat superior nematocidal action, its application is difficult and costly. Therefore, as the dichloropropene-dichloropropane mixture was almost as effective and is more easily applied, it seems that the use of this material might contribute to more satisfactory establishment and growth of peach replants. As it appears that nursery stock produced in Ontario

may carry *P. penetrans* into the orchard, the reintroduction of the parasite in infested nursery stock may be a problem if growers are advised to fumigate the soil before replanting. It would seem advisable that peach nursery stock be grown in fumigated soil or in soil free of plant parasitic nematodes.

When trying to establish the potential nematode pathogens associated with degenerative diseases of perennial crops the importance of the time of sampling is evident. Reference to Fig. 3 shows that sampling only at the end of the second growing season might lead one to believe that ectoparasites are likely to be of great importance in the problem. It is essential, when investigating root rots, to realize the importance of studying closely those organisms associated with the very early phases of lesion formation.

It appears that in a typical replanted orchard the first roots produced by the young tree are attacked within a relatively short time by the endoparasite *P. penetrans* and, during the following few weeks while the soil temperatures remain relatively cool, the nematode propagates rapidly in the newly formed succulent tissues. Throughout this early spring period, as was shown by Koch (1), degeneration of the root system frequently becomes evident. With the onset of higher soil temperatures, the populations drop rapidly, but they increase to a limited extent with the return of cooler soil temperatures in the fall.

As the root system declines the succulent tissues forming the necessary substrate for the endoparasite decay, with the result that the populations of *P. penetrans* decline. Concurrently, forms begin to appear which possess a relatively massive stylet, presumably capable of penetrating suberized tissues and feeding directly in the conductive elements of woody roots.

Thus the ectoparasites, particularly *Paratylenchus*, usually appear in the root zone several weeks after the nursery stock is planted. Their populations increase relatively slowly so that, by the end of the first year, they are still greatly outnumbered by the endoparasite. During the second growing season the population of the endoparasite continues to decline, and the populations of the ectoparasites to increase. Also new forms appear, so that the ectoparasites frequently become the predominant forms in the faunal complex.

Although large populations of *P. penetrans* appear to be connected with the failure of replanted peach trees, the precise role of this nematode in the etiology may only be determined by laboratory studies. Theoretically, *P. penetrans* may be related to the problem by one or both of the following means: (1) the nematode may be an incitant of the problem by creating infection courts for certain bacteria and fungi of peach soils and by affording opportunities for production of toxins through breakdown of amygdalin within the roots, but, by itself, doing relatively little damage; (2) the nematode may be a pathogen per se whose endoparasitic activities profoundly affect growth.

The relation of ectoparasitic nematodes, particularly *Paratylenchus* sp. and *Xiphinema americanum*, to root health and growth of peach replants requires clarification. The feeding activities of ectoparasitic nematodes may afford infection courts for bacteria and fungi, as well as provide a mechanism for the

production of toxins in the roots. As the present study indicates that ectoparasites require several seasons to reach heavy populations and, since there is no apparent correlation of distribution and numbers of ectoparasites to the incidence of the problem, it appears, on an *a priori* basis, that their role is more likely to be additive following the severe limiting effects of *P. penetrans*. This may explain why the replant problem is not severe in the Niagara district, in which *Paratylenchus* is much more numerous than *Pratylenchus penetrans*, as pointed out by Mountain and Boyce (3). In the absence of replant mortality in the early seasons, it is possible that the effect of the ectoparasites may be mainly on longevity and productivity of the orchard after establishment.

Acknowledgments

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BOTANICAL DERIVATIVES CONTRIBUTING TO THE STRUCTURE OF MAJOR PEAT TYPES[1]

NORMAN W. RADFORTH AND H. RONALD EYDT

Abstract

Samples of peat from the Fort Churchill area in Manitoba have been investigated in the hope that a quantitative system would be found to explain peat structure. This entailed the development of a method which would allow detailed study of the peat with the organic elements in their *in situ* position. The quantitative results, categorized according to botanical relationships, were used to indicate the structural differences in the samples studied, and also to elucidate the vegetational succession of the samples. Relationships between subsurface details and surface vegetation are discussed.

Introduction

In recent years, the organic terrain of Canada, commonly referred to as muskeg (2, 3, 4, 5, 6, 7, 8), has received an increasing amount of attention because of intensified northern development. In this connection, the requirements of the petroleum industry have served as an example (7). Their most fundamental problem is one of transport where no roads exist. To achieve access most economically over muskeg, a knowledge of the terrain is therefore desirable. Such knowledge is in large part dependent upon an understanding of peat, the component of the terrain which provides the specialized engineering problems.

Before these problems can be answered, it seems reasonable to suggest that the factors controlling peat formation should be understood. In a particular sense it seems important to place emphasis on finding an approach for the interpretation of peat structure. When Raup (9) indicated some of the botanical problems characteristic of the north, he also specified the need for new information on peat. In the present paper, the authors therefore seek to elucidate the botanical basis on which they think peat structure can best be judged.

Two authors, Dachnowski-Stokes (1) and Radforth (5), have attempted to categorize peat character for reference purposes. Although in each case a reference system was derived that was based on natural as opposed to arbitrary relationships, one system (5) recognized more categories than the other and both were based on qualitative difference. The present authors have aimed at quantitative assessment. They have attempted this to avoid empirical assessment of peat characterization as much as possible, and also to rationalize the apparent differences in organization.

In approaching the problem, two circumstances seemed to hold priority over others. One was the need to indicate which elements within the peat provided maximum significance in designating peat character. The other was to illustrate the mechanics by which quantitative data concerning the elements could be associated and rationalized for comparative studies.

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Contribution from the Department of Biology, Hamilton College, McMaster University, Hamilton, Ontario.

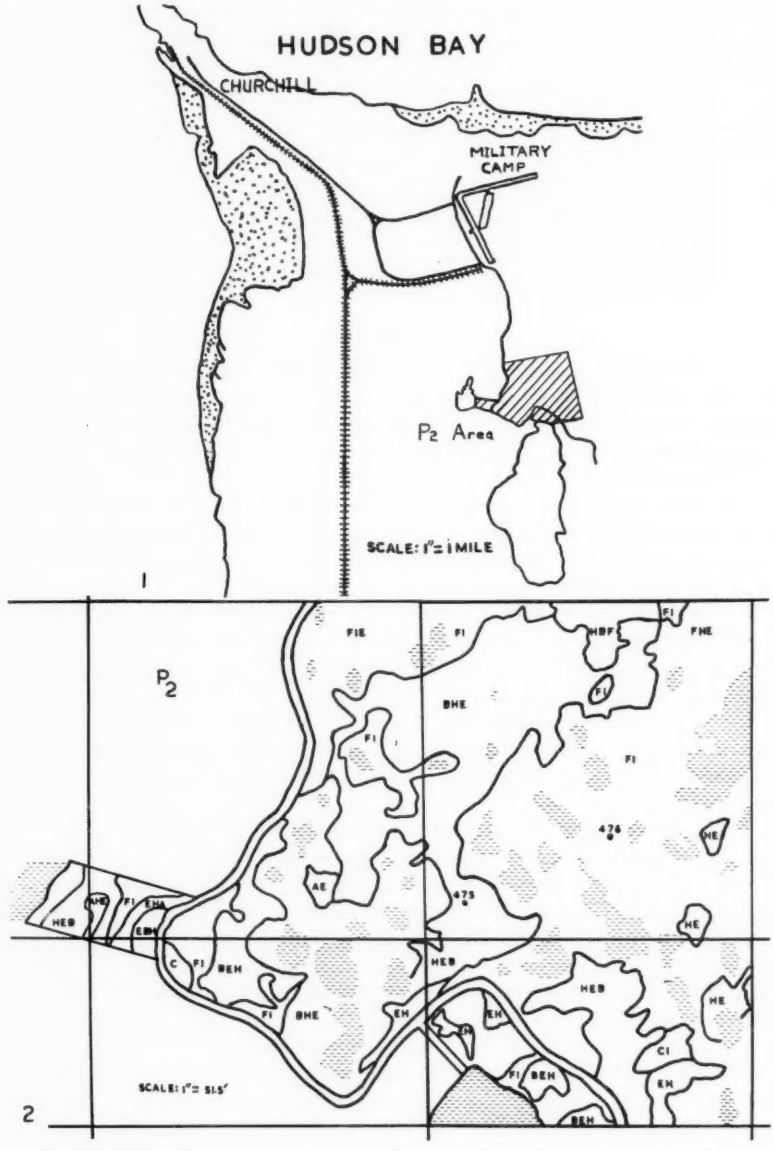

FIG. 1. A map showing the P_2 area in relation to Churchill, Man., and the Military Camp of Fort Churchill.

FIG. 2. A map of the P_2 area showing the surface vegetation classified according to Table I, Fig. 1, and the lakes and ponds.

Radforth (5) described the physical elements which can be seen from a visual examination of peat samples, and which he thought were significant. These are the woody fibrous, non-woody fibrous, and granular constituents. Although the use of these elements in their various proportions is helpful in designating peat types qualitatively, the method has a limitation because quantitative evidence is not available to rationalize the qualitative designations. Further, quantitative evidence is desirable to explain structural trends which the macrofossils in the peat only vaguely signify. Also, if analyses could be made in some way to indicate proportions of the elements, the results would probably be valuable in explaining physiographical differences in the terrain, e.g., drainage patterns.

The development of sample preparations, in which the constituent elements could be clearly observed and readily assessed as to distribution and frequency, was regarded at the outset as the chief subsidiary objective. It was assumed desirable to stabilize the peat so that the macrofossils, with spatial relationships undisturbed, could be examined in the laboratory. Conservation of the natural positions of the macrofossils was thought important not only because of the interrelationships which seemed to be basic for an understanding of peat structure, but also because variation in combinations of macrofossils was assumed to assist in distinguishing peat types.

Development of Method

In order to establish the fundamental steps in the procedure, random selection of peat samples was made from several hundred cores available in the Muskeg Laboratory at McMaster University. To work out refinements in method, two special cores were selected. These represented distinct peat types as classified by Radforth (5), and the field notes for them gave full details on the environment from which they had been removed.

Both cores had been removed from an area of muskeg under ecological investigation (P_2), 4 miles south of Churchill (Figs. 1 and 2). One core (B.475) was removed from an elevated area, the top of which was broken but reasonably level, and which was 10 ft. above the level surface of the meadow-like expanse from which the second core (B.476) was removed. In the former case, the surface vegetation was defined by the formula BHE (*vide* Table IA, where the description for the constituent class letters in the formula are defined). For B.476, the formula characterizing the surface vegetation was FI (Table IB). The peat categories designating structural qualitative value differ; for B.475, the description is coarse-fibrous traversing fine-fibrous (cf. Table IA), for B.476, it is amorphous-granular in fine-fibrous. In one case (B.475), the ground water level was below the mineral sublayer. In the other (B.476), the water level was 2 in. below the surface of the peat.

From each of these cores, a sample, with a surface area of 1 sq. in., was removed from the length of the core. These samples were then divided into 1-in. cubes. They were placed in normal butyl alcohol where they remained for a week. At intervals during this time, they were placed under vacuum

to remove the air, and thus to aid infiltration. Then the cubes were transferred directly into melted paraffin for a week. Again vacuum was used to accomplish the removal of normal butyl alcohol, and its replacement by paraffin. After cooling, the excess paraffin was removed and the cubes of embedded peat were sectioned with a sliding microtome at a thickness of 20μ. Haupt's adhesive was used to fasten the sections to 2 in. by 3 in. glass slides.

TABLE IA

A CHART SHOWING RADFORTH'S (2) CLASSIFICATION OF SURFACE VEGETATION

Coverage type (class)	Woodiness vs. non-woodiness	Stature (height)	Texture	Growth habit	Example
A	Woody	15 ft. or over		Tree form	Spruce, larch
B	Woody	5 to 15 ft.		Young or dwarfed tree or bush	Spruce, larch Willow, birch
C	Non-woody	2 to 5 ft.		Tall, grass-like	Grasses
D	Woody	2 to 5 ft.		Tall shrub or very dwarfed tree	Willow, birch, Labrador tea
E	Woody	0 to 2 ft.		Low shrub	Blueberry Laurel
F	Non-woody	0 to 2 ft.		Mats, clumps or patches, sometimes touching	Sedges Grasses
G	Non-woody	0 to 2 ft.		Singly or loose association	Orchid, pitcher plant
H	Non-woody	0 to 4 in.	Leathery to crisp	Mostly continuous mats	Lichens
I	Non-woody	0 to 4 in.	Soft or velvety	Often continuous mats or hummocks	Mosses

TABLE IB

DESCRIPTIVE TERMS FOR 16 CATEGORIES OF PEAT STRUCTURE AS PROPOSED BY RADFORTH (5)

Category number	Description
1	Amorphous-granular
2	Non-woody fine-fibrous
3	Amorphous-granular in fine-fibrous
4	Amorphous-granular in woody fine-fibrous
5	Amorphous-granular, fine-fibrous, non-woody in woody fine-fibrous
6	Amorphous-granular with woody fine-fibrous held in coarse-fibrous
7	Non-woody fine-fibrous covering amorphous-granular in fine-fibrous
8	Non-woody fine-fibrous; mound in coarse-fibrous
9	Woody fine-fibrous held in coarse-fibrous
10	Woody particles in non-woody fine-fibrous
11	Woody and non-woody particles in fine-fibrous
12	Coarse-fibrous, woody
13	Coarse-fibrous traversing fine-fibrous
14	Non-woody and woody fine-fibrous held in coarse-fibrous
15	Woody with amorphous-granular in fine-fibrous
16	Woody coarse-fibrous with scattered woody erratics

The slides were placed on two glass rods which were across the bottom of Petri plates. To remove the paraffin, xylol was added to the plate by means of a pipette until the slide was covered. After 10 minutes the level of the xylol was lowered with the aid of a pipette. Mounts were completed in Canada balsam.

Results

Before and after sectioning, no loss of material through fragmentation could be appreciated. During the process of infiltration the cubes retained their original shape, and no lacunae or distortion was detected subsequently in any of the sections. Overlap of the components in all sections was minimized and the optical conditions were such that optimum clarity was afforded. On this basis the method of preparing the material for examination was therefore satisfactory and the infiltration procedure in particular was effective.

From a superficial examination of the peaty elements on the slide, it was apparent that structural properties of the samples varied with respect to density. This effect seems to be a function of frequency and distance apart of the constituent elements. To reveal density differences in measured terms, a Whipple disk was utilized. The squares of the disk were thus superimposed above the image of the fragments in the section as the latter was viewed in the low power of the microscope. The number of squares which covered the peaty elements was taken as the density for that field. Five different fields randomly selected from the mount were examined in this fashion. This procedure was performed 10 times for successive depths of 1 in.; five for the cross section of each depth, and five for the longitudinal section of the sample. The average of these 25 densities was designated as the density index of that mount.

Each mount was then reassessed for density measurements of characteristic constituents of the peat. To acquire the data, the Whipple disk was used as before, but in applying the method, given types of constituents were chosen. Thus, for one set of determinations, the analysis would relate to woody axes, for another, bark and cuticle, for a third, leaves, and for the fourth, amorphous granular micronodules. When lichenous material appeared, its density was also noted. Since each field was completely covered by 100 squares and the derived measurements were fractions thereof, the density indices were percentages of cover for each of the botanical categories named. These indices are shown in Tables II and III. This system of analysis was used for both cores (B.475, B.476).

The selection of names to designate the peaty elements was difficult because of a need to satisfy two situations. First, the material was fragmentary, a condition which was made more difficult by sectioning. This made it practically impossible to name the fragments on a generic basis, and consistent naming was essential to facilitate quantitative estimation. Secondly, the elucidation of vegetal history was important in the full assessment of the peat type of the cores under study. The decision was therefore made to designate the fragments in organographic rather than in generic terms. In this way, all

of the peaty material in a given sample could be accounted for, whereas, had generic designations been attempted, this could not have been achieved. To illustrate this point, the woody stems represented in section (Figs. 16, 19) showing bark and secondary thickenings cannot be assigned taxonomically, but can be used as a characteristic entity for quantitative studies and palaeoecological investigations. The type of fragment represented in Fig. 9, a non-woody stem lacking bark and secondary thickenings, is also unidentifiable generically, but is significant in the same way as the first example with which it obviously contrasts by reason of anatomical difference.

Cross-sections of the cores show that although, in the main, most axes are cut longitudinally, the direction of cut is slightly oblique (Figs. 9 and 10). Conversely, in longitudinal sections, most axes are cut not quite transversely (Fig. 11).

Both cores exhibit this, but they contrast in that each demonstrates its own characteristic peaty elements. In sections of B.476, all the material was non-woody. One of the non-woody characteristics is afforded by the presence of leaves still attached to herbaceous stems (Figs. 9, 10, 11, 13). Because of this, stems and leaves are counted together for density value (Table III). Another feature in sections of B.476 is the presence of non-woody axes with aerenchymatous tissue (Fig. 14). An element which contributes significantly to characterization of the non-woody constitution is shown in Fig. 12.

In B.475, woody material predominated, and was responsible for providing the aspect by which sections could be differentiated from those of B.476. Characteristic of this material was the bark in structural contrast to the xylem forming the resistant inner core. Both cross sections and longitudinal sections of the axes illustrated this (Figs. 15, 16, 19). Leaves were not found in association with woody axes; in fact, leaf elements were seldom seen. An exception to the latter was encountered at 1 in. below the surface of the peat. Evidence of leaf structure found at this level is shown in Fig. 17.

TABLE II

ANALYTICAL DATA RELATIVE TO BORE 475

Depth (in.)	Density (%)	Woody axes (%)	Loose bark and cuticle (%)	Leaves (%)	Lichens (%)	Amorphous granular (%)
0–1 c.s.	18	1	5	—	12	—
0–1 l.s.	43	12	13	—	18	—
1–2 c.s.	34	21	6	7	—	7
1–2 l.s.	40	17	9	14	—	17
2–3 c.s.	43	36	7	—	—	17
2–3 l.s.	41	29	12	—	—	29
3–4 c.s.	41	35	6	—	—	16
3–4 l.s.	48	34	14	—	—	50
4–5 c.s.	45	30	15	—	—	42
4–5 l.s.	53	33	20	—	—	63
5–6 c.s.	60	45	15	—	—	60
5–6 l.s.	77	63	14	—	—	70

NOTE: c.s. =cross section; l.s. =longitudinal section.

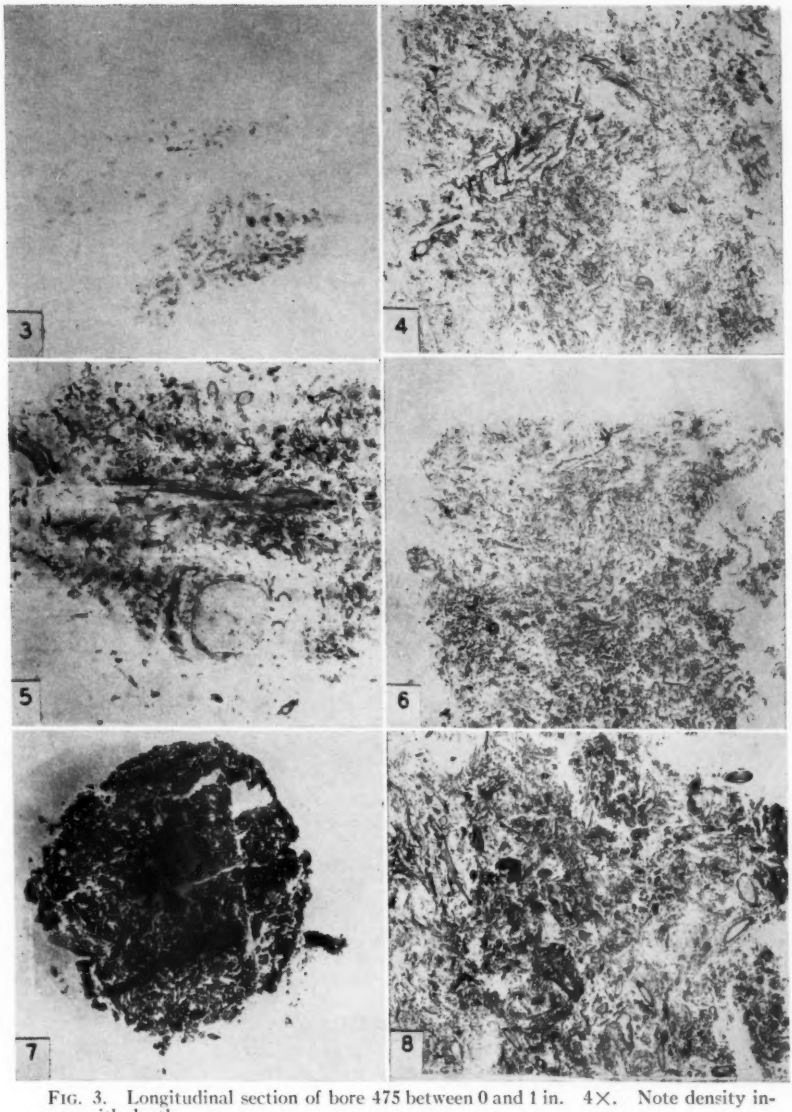

FIG. 3. Longitudinal section of bore 475 between 0 and 1 in. 4×. Note density increase with depth.

FIG. 4. Cross section of bore 476, 5 in. below surface of muskeg. 4×.

FIG. 5. Longitudinal section of bore 475. 4×. The relative abundance of cross sections of woody axes should be noted.

FIG. 6. Longitudinal section of muskeg bore 476 from 3 to 4 in. below surface. 4×. Note the increase of density toward the lower portion of the figure.

FIG. 7. Cross section of bore 475 at depth of 4 in. 4×. Note dense woody material.

FIG. 8. Cross section of bore 476. 4×. Note the crowdedness of the axes due to compaction and the interstices which are filled with leaves.

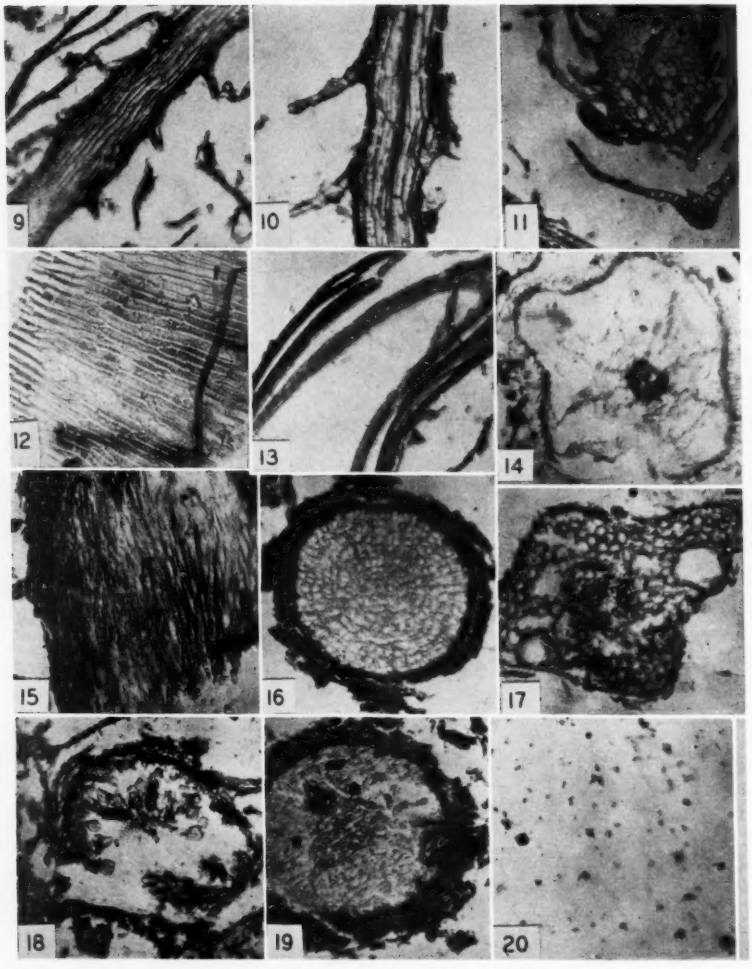

FIG. 9. Longitudinal section of non-woody axial material showing cellular construction. 67×. Bore 476, depth 4 in.
FIG. 10. Longitudinal section of non-woody axis. 67×. Depth 1 in.
FIG. 11. Non-woody stem with branches. 67×. Bore 476, depth 1 in.
FIG. 12. Detail of non-woody leaf. 326×. Bore 476, depth 2 in.
FIG. 13. Oblique section of non-woody leafy material. 67×. Bore 476, depth 3 in.
FIG. 14. Cross section of water plant stem. 67×. Bore 476, depth 3 in.
FIG. 15. Longitudinal section of woody material. 67×. Bore 475, depth 3 in.
FIG. 16. Cross section of woody axis. 67×. Bore 475, depth 5 in.
FIG. 17. Cross section of spruce leaf. 67×. Bore 475, depth 1 in.
FIG. 18. Group of pollen grains. 67×. Bore 475, depth 3 in.
FIG. 19. Cross section of woody axis. 67×. Bore 475, depth 3 in.
FIG. 20. Amorphous-granular particles. 67×. Bore 475, depth 6 in.

Pollen grains and spores occurred singly and in clumps (Fig. 18). However, in relation to peat structure, they were ineffectual because of their small size and relatively small numbers. Therefore, the occurrence of pollen grains and spores was not used in the quantitative determination of the cores examined, even though their presence was encountered throughout the peat.

The amorphous granular micronodules occurred commonly and in large numbers in both cores. Their size ranged from 1 μ to 25 μ, a condition which can be appreciated from examination of Fig. 20. These particles were outstanding because of their large numbers and lack of botanical structure. Because of their small size and lack of botanical character, it was difficult to regard them on a comparative basis with any of the other peat elements. On the other hand, the importance of the amorphous organic micronodules as structural entities could not be rejected. Therefore, density measurements have been made of the amorphous granular component (Table II, column 7, and Table III, column 4), and of the total material which was not granular (Tables II and III, column 2). If at least 25% of a square of the Whipple disk (cf. p. 157) was occupied by micronodules, it constituted one density value. The density measurements in Table II, column 7, and Table III, column 4, when compared with those of Tables II and III, column 2, show the degree of influence of nodular to non-nodular units in the structure of the peat.

TABLE III

ANALYTICAL DATA RELATIVE TO BORE 476

Depth (in.)	Density (%)	Non-woody stems and leaves (%)	Amorphous granular (%)
0–1 c.s.	27	27	0
0–1 l.s.	25	25	0
1–2 c.s.	30	30	8
1–2 l.s.	62	62	20
2–3 c.s.	69	69	35
2–3 l.s.	38	38	30
3–4 c.s.	78	78	30
3–4 l.s.	69	69	30
4–5 c.s.	81	81	30
4–5 l.s.	69	69	30
5–6 c.s.	70	70	40
5–6 l.s.	69	69	40
6–7 c.s.	73	73	45
6–7 l.s.	75	75	50
7–8 c.s.	86	86	50
7–8 l.s.	89	89	80
8–9 c.s.	93	93	85
8–9 l.s.	95	95	90

NOTE: c.s. = cross section; l.s. = longitudinal section.

The lichenous matter in B.475 provided the major component of the structure of the peat in the top layer. This material was loosely associated, and though it was very abundant in the upper inch of B.475, it was lacking elsewhere.

Discussion

The experimental limitation that the peat elements must be in their *in situ* positions when examined on the slides imposed strict requirements on every aspect of the method. The choice of normal butyl alcohol, in combination with dry samples and liquid paraffin, kept the embedding procedure to a minimum of time. Because the normal butyl alcohol softened the peat, and penetrated the lacunae, the paraffin replacement was facilitated and therefore hastened. Thus, the danger of displacement due to lengthy transfers was minimized. If fragmentation from the use of the vacuum pump occurred, it was not observed, nor was any distortion as the paraffin hardened. Finally, the softening process discouraged fragmentation which might have led to serious misconceptions in identification of elements and in quantitative determination.

The orthodox method of removing the paraffin from the sections was not effective in this problem. When the slides were dipped into a xylol bath and then removed, the force of the surface tension was greater than that of the adhesive holding the material to the slide. To overcome this difficulty, the method was devised whereby xylol was added and removed from a stationary slide. When the mount was applied to the fluid, the arrangement of the particles was completely disrupted, but when the reverse procedure was applied, the particles retained their natural orientation.

The use of the Whipple disk made it possible to assess density and botanical identity relative to a precise location within the peat sample. Thus, comparison of structure and botanical affinity was correlative, which was essential to achieve the aims of this work.

From examination of Tables II and III, it is apparent that density increased with depth. It is perhaps obvious that mechanical compaction, in part, explains this, but it may also be claimed that density increase can be referable to the assemblage of botanical derivatives for explanation, e.g., masses of woody axes.

Figs. 3, 5, and 7 show the density increase noted in B.475. The uppermost section (Fig. 3) shows the extremely small amount of organic material detected on the slide. There is a rapid increase in density in the second inch (cf. Table II and the photograph of a longitudinal section, Fig. 6) where there is a distinct difference between the upper and lower edges. Density increase also occurs in B.476, and is illustrated in a comparison of the photographs (Figs. 4, 6, and 8).

The axes, both woody and non-woody, formed a skeletal network around which the other elements were arranged. From Tables II and III, it is obvious that the density index for the axes greatly affects the total density. Also qualitative examination showed that as axis material dominated the peat in

amount (not necessarily numbers of axes) it also provided mechanical continuity in the structural sense. Thus, the axes played a major role, the other elements a minor, in effecting peat synthesis.

While the roles played by the elements in the peat synthesis can be shown quantitatively and qualitatively, it can be stated only qualitatively that where axes fractured with increasing depth, the non-woody ones fractured more than the woody. This is a feature of significance wherever mechanical properties of the peat are to be assessed.

The density index between B.475 and B.476 showed a variation which might be explained by the fracturing of the non-woody axes. Because the axes in B.476 fracture more readily, compaction can take place more easily. Thus, density indices could be higher, which they were.

Apparently, fragmentation of fractured axes in B.476 contributes to the filling of the interstices in the skeletal system. Also, leaves contribute to the contents of the interstices more readily here than does bark and cuticle. The reverse is true in B.475 where bark and cuticle are prominent. Therefore the peat type of the two cores can be readily distinguished by reference to axes, and to the components which fill the interstices among the axes.

It will be appreciated that the quantitative analysis has afforded a means for differentiating fundamental factors in peat construction. However, full value from these comparisons cannot be appreciated entirely until the significance of the elementary botanical constitution has been examined. To facilitate this, further consideration has been given to the relative importance of elements other than axes.

In B.475, leafy fragments rarely occurred at depths beyond the first inch. Their apparent absence suggested fragmentation, a circumstance which presumably would be accompanied by increase in the amorphous granular component. Such increase did in fact occur (cf. Table II) and the density with reference to this component for the 5-in. depth was greater in B.475 than in B.476. However, the effect of fragmentation was offset by the high incidence of axial elements in the case of B.475. Thus, density values contribute to an understanding of the structural composition of the peat, mainly when they are considered on the background of botanical synthesis. This combination of density and botanical affinity accounts for a degree of resilience in this peaty sample which was not encountered in others from B.476. It also suggests an important factor which must be considered when mechanical properties of peat are to be assessed.

In B.476, the non-woody leaves, persistent to the lowermost depths of the peat, shared prominence with high density in amorphous granular elements, particularly at levels beyond the fifth inch. The combination here of density and botanical affinity enables the reader to contrast the mechanical potentialities of this peat with that of B.475. As it was true for the latter, so it may be claimed for B.476 that this combination is basic to the characterization of peat construction. Although leaves are found in organic connection with the axes, this feature is significant only for peat identification. It does not signify either resilience or strength in that the leaves and the axes are

non-woody. However, it does contribute to cohesiveness and may have indirect bearing on the rate at which mechanical deterioration of the peat can be expected.

The fact that groups of pollen and spores occurred contributes little to an understanding of peat construction. Therefore, although they were encountered here, their potential in peat characterization has not been exploited (Radforth (5)).

The high predominance of lichenous matter in the upper inch of B.475, and its absence below, suggests that it fragments rapidly. This situation compares favorably with that for leaves in the same core. While it can be claimed that the lichenous component contributes to the granular matrix in the sample, it makes little contribution to peat characterization in the mechanical sense.

The progressive increase in the amounts of amorphous micronodules with depth was common to both cores. It might be suggested that this feature is a result of downward migration of particles as well as physical deterioration. B.475 was removed from a position above the water table; B.476 from a position within the water table. Any claim that the increase in density of amorphous micronodules was the effect of precipitation encouraging downward migration is therefore questionable. Thus it would seem that the disposition of the other elements in the peat or some other internal factor rather than an external agency controls the distribution of the amorphous granular particles.

A quantitative approach to peat examination, if adequate, might be expected to contribute in specific ways to the solving of several fundamental problems centering on structural interpretation. This possibility, as an extension to the conclusions reached in the present discussion, is under consideration by the writers, and is for future report. Two fundamental applications, however, seem to belong more properly to this account. One concerns orientation of the major components in the peat type represented in B.475; the other has reference to the question of relationships between peat structure and vegetal cover.

In B.475, the quantitative approach not only revealed a means for assessing comparative densities of the peat components, but also led to the definition of the major component, the woody axes, and how these were largely oriented in the horizontal plane. A consideration of how mechanical properties of peats are to be assessed is facilitated by such evidence. For instance, the cohesive potential of the mat produced by peat of the type in B.475, clearly a function of fiber type and orientation as well as density factor, should be measured in accordance with this situation. Also, the evidence suggests that even without measurement, the cohesive strength of the type of peat in B.475 will always be of a higher order than that in B.476, and is much more appropriate as a bearing medium than would be a peat in which the major component was predominantly in the vertical plane. Finally, if shear failure is important as a mechanical factor, density measurements of the horizontally placed component should be significant in analyzing this factor and in predicting planes and patterns of shear failure.

The cover formula defining the vegetation above B.475 is BHE. From this, one might look for all three classes in the formula to dominate as components of the core. On the other hand, B and H, the two most significant factors significant in the formula, were almost completely absent, and E, the least significant in the cover formula, showed higher significance in the core. Where FI was the cover formula (B.476), F, the significant component in the formula, was markedly significant in the core, and I was relatively unimportant. Thus, the contributions from the quantitative approach affords understanding of surface–subsurface relationships to a significant degree in the specific, though perhaps not always positive sense.

The prediction of peat type defined on a qualitative basis made from examination of surface character is often accompanied by uncertainty in the mind of the interpreter. The quantitative approach developed here has apparently justified qualitative designation (Radforth (5)), and has contributed towards the explanation of differences in peat constitution.

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STUDIES OF THE KETO ACIDS OF WHEAT

I. BEHAVIOR DURING GROWTH[1]

R. M. KRUPKA AND G. H. N. TOWERS

Abstract

A survey of the keto acids of wheat showed α-ketoglutaric, pyruvic, glyoxylic, hydroxypyruvic, α-ketoisocaproic, oxaloacetic, and a number of unidentified keto acids to be present. The behavior of α-ketoglutarate, pyruvate, and glyoxylate in germinating seedlings under different conditions in light and dark and in aging leaves and in starving excised leaves was studied. These keto acids appeared and reached a peak during the early stages of germination. Oxaloacetic acid was present only in trace amounts. An unidentified keto acid was found to accumulate in etiolated seedlings. The effect of a nitrogen atmosphere on keto acid levels and the incorporation of glucose-C^{14} into pyruvate, α-ketoglutarate, and oxaloacetate provided evidence for the operation of the tricarboxylic acid cycle in seedlings.

Introduction

The keto acids, particularly pyruvate, oxaloacetate, and α-ketoglutarate, are well-known intermediates in carbohydrate, organic acid, and amino acid metabolism in higher plants. Since they are involved in both anabolic and catabolic pathways, a study of their behavior during growth was considered to be of interest. Analyses were made of keto acids of germinating seedlings, aging leaves, and excised leaves. It was felt that studies of this nature would shed new light on the part played by these compounds during various phases of growth.

Materials and Methods

Plant Material

Thatcher wheat seed was submerged briefly in sodium hypochlorite solution containing 2% available chlorine and a small amount of detergent, washed thoroughly in tap water, and germinated at room temperature on wet filter paper in enamel trays. Two methods of germination were used:

Method A. The filter paper on which the seeds were germinated was moistened, but not flooded, and the tray was covered with a second inverted tray, a narrow gap being left between the two. In this way a high humidity was maintained without preventing air circulation. After 2 days in the dark the covering tray was removed and, if green plants were to be studied, the seedlings were transferred to another room and exposed thereafter to continuous illumination from a bank of fluorescent lamps. The paper was kept moist throughout the germination period.

Method B. Just enough water was added to the trays to submerge the grains. Germination was allowed to proceed either in the light or dark.

Keto Acid Determinations

Plant material (3–5 g. fresh weight) was weighed and ground with a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl and extracted by the procedure already reported (16). Etiolated material was ground with the reagent

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Contribution from the Botany Department, McGill University, Montreal, Quebec.

TABLE I

THE ALKALI-SOLUBLE 2,4-DINITROPHENYLHYDRAZONES FROM WHEAT SEEDLINGS OBTAINED ON CHROMATOGRAPHY IN 9:1:4 TERT-AMYL ALCOHOL : ETHANOL : WATER*

Spot no.	DNP	Color with NaOH	Absorption max. in 1% Na_2CO_3 (mμ)	R_f
1	Unknown	Purple	None	.05
2	α-Ketoglutaric acid	Olive-green	385	.10
3	Oxaloacetic acid	Brown	—	.15
4	Unknown	Violet	573	.23
5	Unknown	Pale purple	—	.28
6	Glyoxylic acid	Brick-red	368	.35
7	Pyruvic acid	Chocolate-brown	376	.41
8	Hydroxypyruvic acid	Orange-yellow	379	.45
9	Unknown	Green	374	.50
10	Unknown	Prussian-blue	—	.53
11	Glyoxylic acid	Red-brown	368	.58
12	Unknown	Light blue	—	.63
13	Unknown	Gray-violet	405	.67
14	Pyruvic acid	Brown	375	.70
15	Unknown	Purple	—	.76
16	α-Ketoisocaproic acid	Fawn	—	.83
17	Unknown	Red-brown	384–400	.96

*A vial of 4% NH_4OH was included in the chromatography chamber.

under a dim red light in the darkroom. Three aliquots of the solution of purified keto acid DNP's* were chromatographed by the ascending method using the upper phase of a 9:1:4 mixture of tert-amyl alcohol:ethanol: water. A vial of 4% NH_4OH was included in the chamber. One lane of the developed chromatogram was cut out and sprayed with ethanolic NaOH since this treatment alters the colors of the DNP's characteristically (see Table I). The spots in the other two lanes, identified by comparison with the sprayed lane, were cut into strips and placed in a 15 ml. test tube together with 5 ml. of 1% Na_2CO_3. The isomeric spots representing one keto acid were determined together after 30 minutes. The eluate was mixed by inverting the stoppered test tube once or twice and its optical density was measured in a Beckman model DU spectrophotometer at the appropriate wave length. α-Ketoglutaric acid DNP was determined at 385 mμ, pyruvic acid DNP at 375 mμ, and glyoxylic acid DNP at 368 mμ. The reproducibility of the method with 3–5 g. of plant material was found to be $\pm$ 10%. The recovery of glyoxylate DNP was 62%, of pyruvate DNP 89%, and of α-ketoglutarate DNP 90%. These recovery values are based on an average of 12 determinations. The values obtained with plant materials were corrected using these recovery factors.

In general, DNP's were identified by (*a*) cochromatography with standard keto acid DNP's,† (*b*) characteristic color of the DNP spot when sprayed with an ethanolic solution of NaOH, (*c*) absorption spectrum of the eluted DNP in 1% Na_2CO_3, and (*d*) chromatography of the amino acid formed on hydrogenolysis of the keto acid DNP (17).

*The abbreviation DNP will be used for 2,4-dinitrophenylhydrazone.

†Pyruvic, oxaloacetic, and α-ketoglutaric acids were obtained from Nutritional Biochemicals Corp., Ohio. Glyoxylic acid was prepared from tartaric acid by the method of Metzler *et al.* (10).

Experiments and Results

1. Identification of Keto Acids

α-Ketoglutaric, pyruvic, glyoxylic, hydroxypyruvic, α-ketoisocaproic, oxaloacetic, and a number of unidentified keto acids were detected in wheat tissues. α-Ketoglutaric, pyruvic, and glyoxylic acids were present in easily detectable amounts in all parts of seedlings except the grain. Hydroxypyruvic acid was identified by its hydrogenolysis products. Two-directional paper chromatography in water-saturated phenol followed by 70% pyridine showed serine, glycine, and alanine to be present in the hydrogenolysis mixture. The glycine probably originated in the small amount of glyoxylic acid DNP present as a contaminant. The presence of alanine is to be expected since serine and alanine are formed in approximately equal quantities when hydroxypyruvic acid DNP is hydrogenated (9). α-Ketoisocaproic acid was identified by cochromatography with synthetic α-ketoisocaproic acid DNP and by chromatography of the hydrogenolysis products. Leucine was the only amino acid obtained on hydrogenation.

Unknown No. 4 (Table I) appeared to be of particular interest because of its response to varying physiological conditions. It was present in much larger amounts in etiolated than in green seedlings, diminished when etiolated seedlings were transferred to the light, and accumulated when green seedlings were transferred to the dark. The effect of a 24 hour period of anaerobiosis was to decrease threefold its concentration in etiolated seedlings. It was rapidly destroyed in aqueous homogenates of the tissue but was stable to boiling 80% ethanol and to grinding in 6 *N* HCl. Unknown No. 4 was isolated, but in insufficient quantity for elemental analysis. Six-day-old etiolated seedlings (2¼ kg.) were macerated in a Waring Blendor with the reagent and the DNP's extracted by the usual procedure. The solution of alkali-soluble DNP's was chromatographed on a cellulose column irrigated with *n*-butanol saturated with 4% NH_4OH. When unknown No. 4 appeared to have separated from adjacent bands, the column was extruded and the portion containing this band cut out. The DNP was eluted with ethyl acetate containing acetic acid and the eluate rechromatographed on paper. The chromatogram was allowed to develop in the solvent for 5 days during which time a number of other bands were resolved from it. The band was cut out, eluted with 1% Na_2CO_3, the solution acidified and re-extracted into ethyl acetate. The substance was rechromatographed on paper and, after elution, less than 2 mg. of a brown non-crystalline material was obtained.

Unknown No. 4, in the acid form, was dissolved in water and titrated against 0.001 *N* NaOH using a Beckman pH meter. The titration curve showed a single major inflection at pH 6.6, at which point the color of the solution, originally yellow at pH 3.6, turned blue.

Part of unknown No. 4 was hydrogenated and the products chromatographed in two solvent systems with amino acid standards. The only ninhydrin reactive substance detected had an R_f of 0.74 in water-saturated phenol compared with 0.37 and 0.57 for leucine and alanine respectively and

an R_f of 0.41 in 4:1:5 butanol:acetic acid:water compared with 0.47 and 0.16 for leucine and alanine. Its movement was unaffected by the presence of $CuCO_3$ on the paper (2). An infrared spectrum of the DNP provided evidence for the presence of carboxyl but not free hydroxyl groups in the molecule. There were broad peaks at 3400 cm.$^{-1}$ and 1725 cm.$^{-1}$ corresponding to absorption by the hydroxyl group and carbonyl bond of the carboxyl group. The blue color given by this compound with ethanolic NaOH suggests that it is a *bis*-DNP so that the parent compound may be a diketo or keto-aldehyde acid with carbonyl groups which are not α to the carboxyl group. By comparison with synthetic DNP's and *bis*-DNP's it was shown that the compound was not diketosuccinic, formylpyruvic, or δ-aminolevulinic acid.

As a further step in the survey of wheat keto acids the alkali-soluble DNP's from 20 g. of etiolated seedlings were hydrogenated and the products chromatographed. The hydrogenolysis mixture gave rise to spots corresponding to glycine, alanine, serine, leucine, aspartic, and glutamic acids, as well as six unidentified spots, one of which corresponded to the hydrogenolysis product of unknown No. 4. Thus a total of 12 amino acids as compared with 17 DNP spots were detected. The discrepancy is due partly or wholly to the formation of isomeric DNP spots.

The presence of aspartic acid in the hydrogenolysis mixture indicates that oxaloacetic acid, though not detectable as a DNP with the amounts of plant material used, is present in wheat seedlings. It was detected as a DNP in first leaves 28 days after germination but not at other times during the aging of the first leaf. That the general absence of oxaloacetate was not due to the method of analysis was shown in a number of ways. When a solution of oxaloacetate was ground with plant material in the presence of the reagent, it was recovered as the DNP with very little loss. The DNP was detectable in other materials such as strawberry leaves in which it has been reported to occur (6) and in the fungus, *Ascocybe grovesii*. Low radioactivity in the position of oxaloacetic acid DNP was detected on radioautographs of young leaves fed with glucose-C^{14}.

A synthesis of keto acids, including oxaloacetate, occurred in aqueous homogenates of young seedlings. Analyses made at intervals after maceration showed that the α-ketoglutarate content doubled in 2 hours, that the levels of glyoxylate and pyruvate increased at first but declined after about 1½ hours, and that oxaloacetate appeared within 10 minutes, reaching a steady level of approximately 20 μg. per g. dry weight. It may be noted that with this treatment unknown No. 4 disappeared immediately. These changes did not take place in boiled homogenates.

In order to test the possibility that keto acids were being formed from corresponding amino acids, glutamic acid-C^{14}, aspartic acid-C^{14}, alanine-C^{14}, and glycine-C^{14} were incubated with homogenates. Glutamic acid, aspartic acid, and alanine gave rise to α-ketoglutarate, oxaloacetate, and pyruvate respectively. Glycine-C^{14} did not give rise to detectable glyoxylate-C^{14}.

2. Changes in Levels of α-Ketoglutaric, Pyruvic, and Glyoxylic Acids during Germination

Keto acids were not present in detectable quantities in the ungerminated grain, and throughout the period of germination the amounts in the grain remained very low (0–8 μg./g. dry weight). The changes during the first 7 days in content of α-ketoglutaric, pyruvic, and glyoxylic acids in degrained

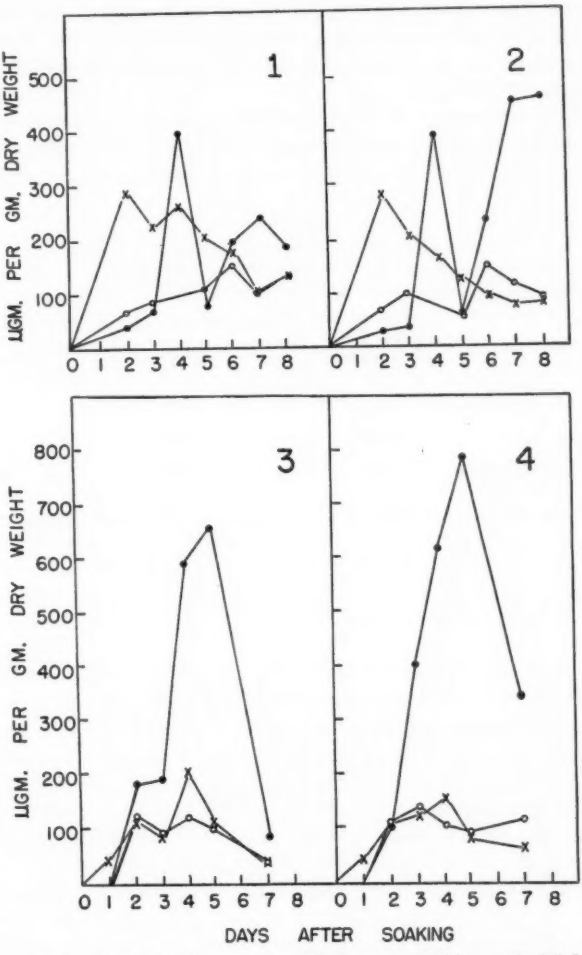

FIG. 1. Levels of keto acids in wheat seedlings germinated by method A in the light.
FIG. 2. Levels of keto acids in wheat seedlings germinated by method A in the dark.
FIG. 3. Levels of keto acids in wheat seedlings germinated by method B in the light.
FIG. 4. Levels of keto acids in wheat seedlings germinated by method B in the dark.
●—● Glyoxylic acid. ○—○ Pyruvic acid. ×—× α-Ketoglutaric acid.

seedlings growing in the light or dark and germinated by method A or B are shown in Figs. 1–4. These keto acids were present in the leaf, root, and coleoptile (see Table II).

3. The Levels of α-Ketoglutaric, Pyruvic, and Glyoxylic Acids during the Aging of the First Leaf

Samples of the first leaf of wheat germinated and grown in soil in the greenhouse during late February and early March were analyzed for keto acids at intervals from 13 to 39 days after planting. At the time when the first sample was taken, the first leaves had reached a stage of early maturity. The results are shown in Fig. 5.

By 28 days after sowing the first outward signs of aging were apparent; the leaves were slightly mottled and their tips were burned. By 35 days the leaves had changed little in appearance, but by 39 days many were dying or

TABLE II

KETO ACID CONTENT OF ROOTS, LEAF, AND COLEOPTILE OF 4-DAY-OLD ETIOLATED WHEAT SEEDLINGS

Organ	Keto acid concentration (μg./g. dry weight)		
	α-Ketoglutaric	Pyruvic	Glyoxylic
Roots	140	270	540
Leaf	330	170	700
Coleoptile	310	380	1300

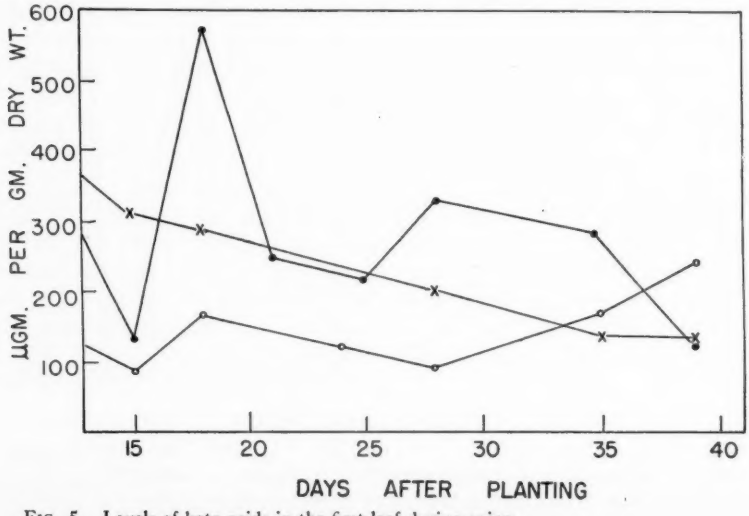

FIG. 5. Levels of keto acids in the first leaf during aging.
●—● Glyoxylic acid. ○—○ Pyruvic acid. ×—× α-Ketoglutaric acid.

dead. The least necrotic of the 39-day-old leaves were used for analysis. All the values in Fig. 5 were calculated on the basis of a dry to fresh weight ratio of 0.1. Actually, this factor increases as the leaf ages (14), so that the values reported here for older leaves may be disproportionately high.

4. The Levels of α-Ketoglutaric, Pyruvic, and Glyoxylic Acids in Serial Leaves before and after Flowering

Samples of the youngest and of the second- and third-youngest leaves of wheat plants grown in soil in the greenhouse were taken not long before the time of flowering and just after flowering, and were analyzed for keto acids. The results are shown in Table III. These values, like those of Fig. 5, were calculated using a dry to fresh weight factor of 0.10. Since the water content of leaves decreases following flowering (14), the values reported for post-flowering leaves may be high.

TABLE III

THE KETO ACID CONTENT (μG./G. DRY WEIGHT) OF SERIAL LEAVES OF WHEAT BEFORE AND AFTER FLOWERING

	α-Ketoglutaric acid		Pyruvic acid		Glyoxylic acid	
Leaf No.*	Before	After	Before	After	Before	After
1	340	570	100	130	430	410
2	300	360	140	210	340	250
3	340	390	240	250	340	270

*Leaf No. 1 refers to the youngest leaf, No. 3 to the oldest.

5. The Levels of α-Ketoglutaric, Pyruvic, and Glyoxylic Acids during Leaf Starvation

First leaves of wheat which was grown in soil in the greenhouse were excised at the ligule when they had reached a stage of early maturity, 13 days after planting. Some of the leaves were analyzed immediately for keto acids. The remaining leaves were rinsed and placed with their bases submerged in distilled water in a beaker in the dark. In order to minimize the danger of bacterial infection, the leaves were rinsed and placed in fresh water each day. Samples were analyzed for keto acids at daily intervals. As in the analyses of etiolated seedlings, the plant material was ground with the reagent under a dim red light in the darkroom. The results of analyses of the excised leaves are shown in Fig. 6.

After 3 days of starvation, the terminal quarter of the leaf was yellow and the subterminal quarter yellowing. After 4 days, three-fourths of the leaf was yellow or yellowing, and after 5 days the leaf was entirely yellow and the terminal 2 to 3 cm. was necrotic.

6. Effect of Nitrogen on Keto Acid Levels

Five-day-old etiolated seedlings were divided into three lots. One was analyzed immediately for keto acids and the second analyzed after a further 24-hours' growth in air. The third was kept under nitrogen for 24 hours in a desiccator and then analyzed. The results are shown in Table IV.

TABLE IV

KETO ACIDS OF 5-DAY-OLD ETIOLATED WHEAT SEEDLINGS MAINTAINED IN AIR AND IN NITROGEN

Treatment	Concentration (μg./g. dry weight)		
	α-Ketoglutaric	Pyruvic	Glyoxylic
5 days air	200	110	460
5 days air + 1 day nitrogen	50	280	1000
6 days air	100	100	550

*7. Feeding of Glucose-1-C^{14} and Glucose-U-C^{14}**

A 0.5 g. quantity of green leaf segments 2–3 cm. long from 3-, 4-, and 5-day-old seedlings germinated by method A were placed in a 25 ml. suction flask. Five microcuries of a solution of either glucose-1-C^{14} or glucose-U-C^{14}, of specific activity 21.7 μc./mg., was added and diluted with water to a final volume of 1.0–1.2 ml. The flask was stoppered and a vacuum applied carefully at the sidearm for a brief period. Upon release of the vacuum the radioactive solution was drawn into the tissue. The tissue was incubated in the dark for 2 hours, preliminary experiments having shown that the total radioactivity of the keto acid fraction of leaves fed radioactive glucose reached a maximum in this time. After being washed with water the segments were analyzed for keto acids. Radioactivity in keto acids was determined by counting the activity of DNP spots on dried chromatograms with a thin end-window Geiger–Müller tube attached to a scaler and by radioautography. The results are shown in Table V.

TABLE V

INCORPORATION OF RADIOACTIVITY INTO KETO ACIDS OF 3-, 4-, AND 5-DAY-OLD WHEAT SEEDLING LEAVES FED WITH GLUCOSE-1-C^{14} OR GLUCOSE-U-C^{14}*

Keto acid	Radioactivity (c.p.m. at the chromatogram surface)					
	3rd day		4th day		5th day	
	Gl–1†	Gl–U‡	Gl–1	Gl–U	Gl–1	Gl–U
Pyruvic	230	350	230	290	150	230
α-Ketoglutaric	300	400	110	80	90	100
Oxaloacetic	Trace§	Trace	Trace	Trace	Trace	Trace
Glyoxylic	0	0	0	0	0	0

*Glucose-U-C^{14} is used as an abbreviation for uniformly labelled glucose-C^{14}.
†Glucose-1-C^{14} .
‡Glucose-U-C^{14}.
§Activity detected only on radioautographs.

Discussion

The Keto Acids of Wheat

The detection of α-ketoglutaric and pyruvic acids in all analyses of wheat is perhaps not surprising, but the explanations for the general absence or low level of oxaloacetic acid, and the high levels of glyoxylic acid often encountered, are not obvious. It appears to be quite possible that oxaloacetate may occur as widely in wheat tissues as do α-ketoglutarate and pyruvate, but that it is not ordinarily detected because of its low concentration. The presence of aspartic acid, the corresponding amino acid, among the hydrogenolysis products of the DNP's supports this idea.

The conversion of aspartate-C^{14} to oxaloacetate-C^{14} in aqueous homogenates shows that the amino acid is one source of the additional keto acid. Since this accumulation is prevented by boiling the homogenate, the conversion is probably enzymatic. Such conversions of amino acids to keto acids are well known. For example, *o*-benzoquinone catalyzes the non-enzymatic deamination of glycine (8), and mixtures of glycine or alanine with catechol and phenolase release glyoxylate. This reaction may be compared with the effect observed by Isherwood and Niavis (6). It would appear that the increases in the quantities of pyruvate, oxaloacetate, and α-ketoglutarate in homogenates are possibly due to such deaminations. Although glycine is known to be deaminated more rapidly than other amino acids in such quinone-catalyzed reactions, the results of the present studies indicate that glycine-C^{14} did not give rise to glyoxylate-C^{14}. The mechanism of the production of additional quantities of keto acids in homogenates remains, therefore, uncertain.

Consideration of the equilibria of certain reactions of oxaloacetic acid may help to explain its low concentration in wheat tissues. The conversion of oxaloacetate to malate (malic dehydrogenase), to citrate (condensing enzyme), or to pyruvate and carbon dioxide (oxaloacetic decarboxylase) proceeds with a decrease in free energy of 7.1, 7.7 (12), or 5.5 (3) kcal. respectively, indicating that the reactions away from oxaloacetic acid are favored. Thus the concentration of oxaloacetic acid in equilibrium with other acids of the tricarboxylic acid cycle and with pyruvate would be very low. It follows that large amounts of this acid are not mandatory in tissues in which the tricarboxylic acid cycle is highly active. The demonstration of radioactivity in oxaloacetic acid in 3-, 4-, and 5-day-old leaves fed glucose-C^{14} (Table V) indicates that oxaloacetate is probably involved in the oxidative degradation of glucose.

The keto acid content of the ungerminated grain was shown to be very low, a result which was not unexpected since the respiratory activity is known to be low (7). Even during germination, when its food reserves were being mobilized and transported to the embryo, the levels of keto acids in the endosperm remained extremely low. In contrast, in the cotyledons of the peanut seedling, in which a similar mobilization and export of food reserves to the growing regions of the embryo takes place, there is as great an increase in keto acid content as in the rest of the embryo (18).

A characteristic feature of the keto acids of germinating wheat seedlings was a rapid increase in their concentrations followed by a decline. Similar observations on other substances including both a variety of substrates and enzymes have been made (5, 11, 13, 15). The sudden and seemingly inconsistent development of certain enzymes associated with glycolysis and the tricarboxylic acid cycle (13) would be expected to affect the concentrations of intermediates in the reaction pathways which they catalyze. Thus if substrates and enzymes required in the synthesis of a compound were readily available, while enzymes catalyzing its destruction were not, the compound might accumulate. This tendency might be exaggerated where large amounts of certain substrates, such as sucrose, are on hand. The work of Oota *et al.* (13) also suggests that increases in the activities of enzymes are due to a liberation rather than a synthesis *in toto*. Such unmasking may set up waves of disturbances in related or dependent substances. As these unmasking processes come to a halt, control mechanisms might exert their effects and a steady level of substrates obtain.

The behavior of α-ketoglutarate during germination may best be explained by such an interpretation. The results of the feedings with glucose-C^{14} show that the relative activity of α-ketoglutarate to pyruvate was much higher (>1) on the third than on the fourth and fifth days ($\backsim\frac{1}{2}$). Since the rate of respiration continues to rise on the third, fourth, and fifth days (7), the content of α-ketoglutarate does not reflect the respiratory activity of the tissues directly. The accumulation of this acid therefore may be due to a blockage of its conversion to succinyl-CoA or to glutamic acid.

The time of the maximum in the content of α-ketoglutaric acid depended on the method of germination. With method B, where the grains were submerged in water, the time of this maximum was several days later than with method A. It is known that prolonged soaking gives rise to high R.Q.'s (7) so that the delayed rise in α-ketoglutarate content probably reflects the inaccessibility of oxygen. The results presented in Table IV show that the α-ketoglutarate content of seedlings placed in an atmosphere of nitrogen decreased markedly, a result which is in accordance with the delayed maximum resulting from prolonged soaking at the start of germination. Further, in the nitrogen atmosphere, pyruvic acid accumulated and, correspondingly, during germination, the peak in pyruvate concentration was reached earlier in the soaked than in the non-soaked grains. It seems likely therefore that the behavior of the keto acids in seedlings germinated by method B reflects a respiratory disturbance.

Attention should be drawn to the remarkable behavior of glyoxylic acid. Although not present in the dry grain, it appeared during germination and reached a maximum level which was nearly twice as high in plants germinated under conditions B (prolonged soaking) as under conditions A. That this effect of method B may be the result of oxygen shortage was shown by the nearly twofold increase in glyoxylate in seedlings maintained under nitrogen for 24 hours (see Table IV). It was shown that glyoxylic acid reached a very

high level in excised leaves (Fig. 6) and that it is not formed from glucose-C^{14} (Table V). The origin of glyoxylic acid and its metabolism in wheat seedlings will be discussed in a later communication.

The changes in keto acid levels in the light and in the dark were similar, indicating that respiration in the early stages of growth is dependent on food reserves of the grain rather than on the products of photosynthesis.

The results of the keto acid analyses of germinating seedlings, the incorporation of radioactivity into pyruvate, α-ketoglutarate, and oxaloacetate when seedlings were fed glucose-C^{14}, and the effect of nitrogen on the keto acid levels of seedlings, taken together, strongly suggest that the tricarboxylic acid pathway is operative in germinating wheat. The higher radioactivity in pyruvic acid in leaves fed with glucose-U-C^{14} as compared with glucose-1-C^{14} (Table V) indicates the operation of some pathway for the dissimilation of glucose to pyruvate other than the Embden–Meyerhof–Parnas pathway of glycolysis. There is evidence for the participation of the Direct Oxidation pathway in the breakdown of glucose in higher plants (1, 4).

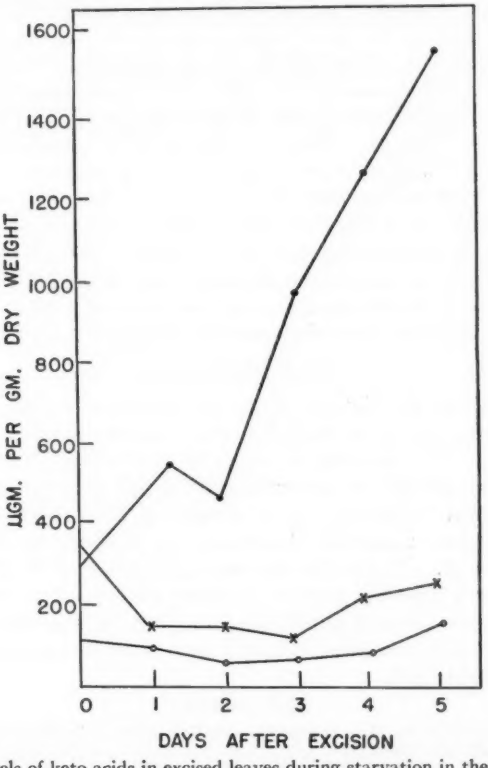

FIG. 6. Levels of keto acids in excised leaves during starvation in the dark. ●—● Glyoxylic acid. ○—○ Pyruvic acid. ×—× α-Ketoglutaric acid.

In spite of major biochemical transformations involving respiration and nitrogen metabolism which accompany the aging of leaves, no obvious correlations of keto acid levels with these changes were apparent. Webb and Fowden (18) found that the α-ketoglutarate content of leaves and roots of the peanut plant rose steadily during development and believed this was related to protein metabolism. Fig. 5 shows that the α-ketoglutarate content of aging wheat leaves declined steadily. Table III shows that the α-ketoglutarate content is unaffected by flowering. The idea that the α-ketoglutarate level is an index of protein metabolism would therefore appear to be incorrect. This is shown even more clearly with excised leaves.

One of the well-known effects resulting from the maintenance of excised leaves in water in the dark is the hydrolysis of protein, with the accompanying accumulation of asparagine and glutamine, and possibly the oxidation of the deaminated residues of amino acids. These changes probably involve keto acids, especially α-ketoglutarate and oxaloacetate, which are related to the accumulating amides. It is somewhat surprising then that the levels of α-ketoglutarate, pyruvate, and oxaloacetate appeared to be unresponsive to these conditions.

While the effects of starvation on the concentrations of α-ketoglutarate and pyruvate are not pronounced, its effect on the concentration of glyoxylate is. The level of glyoxylic acid rose more or less steadily to a very high level on the fifth day (Fig. 6). This behavior is to be contrasted with that in aging leaves (Fig. 5) where the level of glyoxylic acid descended as time went on.

A danger in work with excised leaves is bacterial infection, especially during the latter stages. Such infection might obscure the actual changes taking place and give rise to distorted analytical results. It seems unlikely however that this accounts for the results obtained here, since the levels of α-ketoglutarate and pyruvate remained more or less the same until the end, and since the upward trend in the level of glyoxylate was apparent from the start.

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STUDIES OF THE KETO ACIDS OF WHEAT

II. GLYOXYLIC ACID AND ITS RELATION TO ALLANTOIN[1]

R. M. KRUPKA AND G. H. N. TOWERS

Abstract

In order to determine the metabolic origin of glyoxylate 12 different carbon-14 labelled compounds, including sugars, organic acids, and amino acids, were fed to wheat tissue. With the exception of glycine none gave rise to glyoxylate. The labelling from glycine was slight and could not have accounted for the large amounts of glyoxylate in the tissue. Of a number of inactive compounds fed to degrained seedlings only allantoin induced an increase in the concentration of glyoxylate. A nitrogen atmosphere was found to increase the content of glyoxylate while decreasing that of allantoin. Allantoin-C^{14} was synthesized much more readily from glycine-C^{14} than from glyoxylate-C^{14}. When seedlings previously fed glycine-C^{14} in air were transferred to nitrogen, considerable radioactivity appeared in glyoxylate. These results suggest that glycine is a precursor and glyoxylic acid a hydrolysis product of allantoin.

Introduction

The role of glyoxylic acid in higher plants is not well understood. Attention was drawn to this acid by its behavior in several experiments with wheat (9). During the early stages of germination of wheat the level of glyoxylate rose to a very high value. Its concentration in germinating seedlings and in aging leaves varied widely from day to day suggesting that it may be rapidly formed and dissimilated. The level was doubled in seedlings under nitrogen for 24 hours. The effect of leaf excision and starvation was to produce an even greater increase. The level attained, nearly 1600 μg. per g. dry weight, was about twice that ever attained normally. These findings have prompted a study of the metabolic relations of glyoxylic acid in wheat.

Materials and Methods

Plant Material

Thatcher wheat was submerged briefly in sodium hypochlorite solution containing 2% available chlorine and a small amount of detergent, washed thoroughly in tap water, and germinated at room temperature on wet filter paper in enamel trays.

Keto Acid Determinations

Keto acids were fixed as their 2,4-dinitrophenylhydrazones and determined by a method already described (9).

Carbon-14 Techniques

Glyoxylic acid-1,2-C^{14} was synthesized by the electrolytic reduction of oxalic acid-C^{14} (10). All other C^{14}-labelled compounds were purchased from Atomic Energy of Canada, Ltd.

Carbon-14 was supplied to the plant tissue either as carbon dioxide or as an organic compound such as glucose. The former was supplied as a gas by means of the following technique. A solution of $Na_2C^{14}O_3$ contained in

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Contribution from the Department of Botany, McGill University, Montreal, Quebec.

the sidearm of a small evacuated chamber released gaseous $C^{14}O_2$ when acidified with 1% perchloric acid. When air was admitted through the sidearm, the $C^{14}O_2$ was swept into the main compartment of the vessel which enclosed the seedlings.

The radioactive organic compounds were supplied in solution to the roots of whole seedlings or alternatively to leaf segments. With leaf segments the method of vacuum infiltration was used. In this procedure leaf segments were suspended in a solution of the radioactive compound contained in a 25 ml. suction flask. The flask was stoppered and a vacuum was carefully applied at the sidearm for a brief period. Upon release of the vacuum, the radioactive solution was drawn into the tissue.

The fate of the C^{14}-labelled substances in the living tissues was investigated by radioautography of the chromatographically separated keto acid 2,4-dinitrophenylhydrazones.

The Quantitative Analysis of Allantoin and Allantoic Acid

The plant material to be analyzed (4–8 g.) was extracted twice by refluxing for 30 minutes with 80% ethanol. After removal of most of the ethanol at room temperature under an air jet, the extract was made to pH 10.0 with concentrated aqueous sodium hydroxide and placed in a boiling water bath for 10 minutes. It was then cooled in an ice bath. One-half of this alkaline hydrolyzate was treated with 4 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl. The solution was extracted with ethyl acetate 3 minutes after addition of the reagent, and glyoxylic acid 2,4-dinitrophenylhydrazone was determined by the quantitative keto acid technique, which involves chromatography (9). The other half of the hydrolyzate was made to pH 2 with concentrated hydrochloric acid, brought to the boil, and then cooled rapidly in an ice bath. This acid hydrolyzate was treated with 2,4-dinitrophenylhydrazine reagent (approximately 1 ml. per g. fresh weight of sample) and glyoxylic acid was determined quantitatively as before. The difference between the quantities of glyoxylic acid released in the acid and basic hydrolyses represented the total glyoxylic acid derived from the hydrolysis of both allantoin and allantoic acid. This quantity of glyoxylic acid was converted to the equivalent quantity of allantoin.

Allantoin was detected after two-directional paper chromatography of 80% ethanol extracts (using phenol saturated with water followed by butanol – acetic acid – water (4:1:5) as solvents) by spraying with Ehrlich's reagent, a solution of 1.0 g. *p*-dimethylaminobenzaldehyde in 200 ml. 95% ethanol and 5 ml. of concentrated hydrochloric acid. With gentle heating of the chromatogram this compound gives a bright yellow color.

Experiments and Results

1. Incorporation of Carbon-14 from Various Radioactive Compounds into Keto Acids

The incorporation of radioactivity into keto acids from various C-14 labelled compounds is shown in Table I. A description of experimental

TABLE I

THE INCORPORATION OF CARBON-14 INTO KETO ACIDS AFTER FEEDING OF PLANT MATERIALS WITH A VARIETY OF C^{14}-LABELLED COMPOUNDS

C^{14}-compound	pH	Plant material	Experimental conditions	α-KG	OA	Pyr.	Gly.	OH-Pyr.	α-KI
Glucose-U-C^{14}	7.0	3-, 4-, and 5-day-old green leaf segments	Dark; 2 hours; vacuum infiltration	+	Trace	+	−	−	+
Glucose-1-C^{14}	7.0	3-, 4-, and 5-day-old green leaf segments	Dark; 2 hours; vacuum infiltration	+	Trace	+	−	−	+
Succinate-1,4-C^{14}	4.0	4-day-old etiolated leaf segments	Dark; 2 hours; vacuum infiltration	+	−	+	−	−	−
Succinate-1,4-C^{14}	4.0	Green leaf segments, 7 days old	Dark; ½ hour; vacuum infiltration	+	−	+	−	−	−
Succinate-1,4-C^{14}	4.0	Etiolated leaf segments, 7 days old	Dark; ½ hour; vacuum infiltration	+	−	+	−	−	−
Succinate-1,4-C^{14}	5.0	Green leaf segments, 11 days old	Dim light; 2½ hours; vacuum infiltration	+	+	+	−	−	−
Pyruvate-1-C^{14}	3.0	Etiolated leaf segments, 6 days old	Dark; 1 hour; vacuum filtration	+	−	+	−	−	−
Pyruvate-1-C^{14}	3.0	Etiolated leaf segments, 6 days old	Dark; 1 hour; vacuum infiltration; N_2 atmosphere	+	−	+	−	−	−
Pyruvate-2-C^{14}	3.0	Etiolated leaf segments, 6 days old	Dark; 1 hour; vacuum infiltration; N_2 atmosphere	+	−	+	−	−	−
Pyruvate-2-C^{14}	3.0	Etiolated leaf segments, 6 days old	Dark; 1 hour; vacuum infiltration; N_2 atmosphere	+	−	+	−	−	−
Acetate-2-C^{14}	5.5	Etiolated seedlings, 3 days old	Light; 2 hours; uptake via roots	+	−	+	−	−	+
Acetate-2-C^{14}	5.5	Etiolated seedlings, 3 days old	Light; 2 hours; uptake via roots; N_2 atmosphere	+	−	+	−	−	−
Glyoxylate-1,2-C^{14}	—	Green leaf segments, 10 days old	Dark; 1 hour; vacuum infiltration	Trace	−	+	+	−	−
Glyoxylate-1,2-C^{14}	—	Green leaf segments, 10 days old	Dark; 19 hours; vacuum infiltration	Trace	−	+	+	−	−
Glycine-2-C^{14}	5.0	Etiolated leaf sections, 3 days old	Dark; 4 hours; vacuum infiltration	Trace	−	+	Trace	−	−
Glycine-2-C^{14}	5.0	Green leaf sections, 11 days old	Dim light; 2½ hours; vacuum infiltration	Trace	−	+	Trace	−	−
Alanine-U-C^{14}	5.0	Green leaf sections, 11 days old	Dim light; 2½ hours; vacuum infiltration	+	−	+	−	−	−
Aspartate-U-C^{14}	5.0	Green leaf sections, 11 days old	Dim light; 2½ hours; vacuum infiltration	Trace	+	+	−	−	−
Formate-C^{14}	—	Green seedlings, 7 days old	Light; 4½ hours, uptake via root	+	−	−	−	−	−
$Na_2C^{14}O_3$	7.3	Green seedlings, 7 days old	Dark; 24 hours, uptake via roots	−	−	−	−	−	−
$C^{14}O_2$	—	Green seedlings, 6 days old	Light; ½ hour, photosynthesis	+	Trace	+	−	−	−
$C^{14}O_2$	—	Green seedlings, 8 days old	Light; 126 hours, photosynthesis	+	Trace	+	−	−	+

NOTE: + Indicates radioactivity, and − no radioactivity on radioautographs.
-KG = -ketoglutarate; OA = oxaloacetate; Pyr. = pyruvate; Gly. = glyoxylate; OH-Pyr. = hydroxypyruvate; -KI = -ketoisocaproate.

conditions is included in the table. Many compounds, including glucose-U-C^{14},* glucose-1-C^{14}, succinate-1,4-C^{14}, pyruvate-1-C^{14}, pyruvate-2-C^{14}, acetate-2-C^{14}, glyoxylate-1,2-C^{14}, glycine-2-C^{14}, alanine-U-C^{14},* aspartic acid-U-C^{14},* and $C^{14}O_2$ fed to green leaves in the light, gave rise to labelled α-ketoglutarate and pyruvate. None gave rise to labelled glyoxylic acid, except glycine-C^{14}. Even with the latter, however, the yield of glyoxylate-C^{14} was extremely low, whereas considerable pyruvate-C^{14} was produced.

2. The Feeding of Metabolites to Degrained Seedlings

Young degrained etiolated seedlings were placed in Petri dishes in the dark with their roots and scutella submerged in a solution of one of a number of organic compounds. Sucrose, glucose, ribose, citric acid, glycolic acid, glycine, and allantoin were fed in several experiments. After 18 to 48 hours of feeding, the glyoxylate content of the seedlings was determined quantitatively. The results, presented in Table II, show that only allantoin induced significant increases in the level of glyoxylic acid. Seedlings without the grain removed, which were taken from the same batch of plant material as that used in the feedings of experiment No. 2 (Table II), were analyzed at the beginning of the experiment (when they were 3 days old) and at the end of it (when they were 5 days old). The content of glyoxylate was found to be 660 and 390 μg. per g. dry weight, respectively. The rising glyoxylate content in seedlings fed allantoin therefore does not represent a natural tendency at this stage of development. On the other hand, removal of the grain, or the effects of glycine or water, appeared to slow somewhat the natural decline in the level of glyoxylic acid.

TABLE II

THE GLYOXYLATE CONTENT (μG./G. DRY WEIGHT) OF DEGRAINED ETIOLATED SEEDLINGS AFTER FEEDING WITH SOLUTIONS OF VARIOUS COMPOUNDS

Expt. No.	Compound fed	Concn. (%)	pH of soln.	Initial age of seedlings (days)	Duration of feeding (hr.)	Glyoxylate content
1	Glucose	1.0	7	3	18	390
	Glycolate	1.0	7			440
	Allantoin	0.06	7			1000
2	Water	—	7	3	48	560
	Glucose	1.0	7			430
	Ribose	1.0	7			280
	Glycine	1.0	7			540
	Allantoin	0.06	7			1200
3	Water	—	7	3	48	800
	Ribose	1.0	7			720
	Allantoin	0.06	7			1080
4	Sucrose	1.0	7	3½	24	450
	Citrate	0.5	5.5			180
	Glycolate	0.5	5.5			200
5	Sucrose	0.5	7	4	24	370
	Citrate	0.5	5.5			280

*Compounds uniformly labelled with carbon-14 are indicated by the symbol -U-C^{14}.

3. The Effect of Nitrogen on the Allantoin Content of Young Seedlings

The demonstration that of a number of metabolites fed to degrained seedlings only allantoin induced increases in the level of glyoxylic acid prompted further investigation of the relationship between these compounds.

Four-day-old green seedlings were enclosed in a desiccator containing a nitrogen atmosphere. Allantoin and glyoxylic acid were determined at the beginning of the experiment and after 24 hours growth in nitrogen. The results are shown in Table III. Similar results were obtained in a second experiment.

TABLE III

CHANGES IN THE LEVELS OF ALLANTOIN AND GLYOXYLIC ACID IN 4-DAY-OLD SEEDLINGS DURING ANAEROBIOSIS

Time under nitrogen (hr.)	Glyoxylic acid (μg./g. dry weight)	Allantoin (μg./g. dry weight)
0	295	3300
24	720	1450

4. The Synthesis of Allantoin-C^{14} from Glycine-C^{14} and Glyoxylic Acid-C^{14}

In an experiment designed to elucidate the relation of glyoxylic acid and glycine to allantoin biosynthesis, the roots and scutella of 3-day-old degrained green seedlings were submerged in a solution of glyoxylate-1,2-C^{14} or glycine-2-C^{14} in 0.01 *M* potassium phosphate buffer at pH 5.6. Feeding proceeded in the dark for 24 hours, after which the seedlings were transferred to water in the light for an additional 33 hours. The 80% ethanol-soluble fractions of the seedlings were chromatographed two-directionally using water-saturated phenol followed by butanol – acetic acid – water (4:1:5) as solvents. Radioautographs were prepared.

In the 80% ethanol-soluble fraction of seedlings fed glyoxylate-C^{14}, allantoin was about twice as radioactive as any of a number of other compounds including glycine. In the glycine-C^{14} feeding, most of the radioactivity was found in glycine and allantoin. Radioactivity determinations made by direct counting at the surface of the chromatogram showed that 5.8% of the radioactivity of the 80% ethanol-soluble fraction from the glyoxylate-C^{14} feeding and 14% of that from the glycine-C^{14} feeding was in allantoin.

5. The Effect of Nitrogen on the Formation of Glyoxylate-C^{14} from Glycine-C^{14}

It has been shown in the preceding sections that glycine is involved in the synthesis of allantoin. It was also shown that the allantoin content of seedlings decreased under nitrogen with a concomitant increase in the level of glyoxylate. In order to determine if the additional glyoxylate which appeared during anaerobiosis was produced at the expense of allantoin, seedlings were fed glycine-C^{14} for 24 hours and then placed in nitrogen for an additional 24 hours. At the end of this period the keto acids were analyzed for radioactivity. Under these conditions, glyoxylic acid was found to be the most radioactive of the keto acids.

Discussion

A number of metabolic pathways through which glyoxylate might be derived are known. Glycine may give rise to this compound by transamination (7) or conceivably by oxidative deamination. Glycolic acid oxidase, which catalyzes the conversion of glycolate to glyoxylate is known in higher plants (15). Glycolaldehyde, which has been implicated in the interconversion of sugars, appears to be another possible precursor. Isocitritase, which has been identified recently in the castor bean (8), catalyzes the cleavage of isocitrate to glyoxylate and succinate. Work with fungi has led to the suggestion that acetate is converted to glycolic and glyoxylic acids and then to oxalic acid (1, 13). Allantoin is also a source of glyoxylic acid. Enzymes which convert allantoin to allantoic acid (allantoinase) and allantoic acid to two molecules of urea and one of glyoxylate (allantoicase) have been demonstrated in many higher plants, although not in wheat (14). The reaction sequence is hydrolytic. Allantoin and allantoic acid are known to occur in a wide variety of plants (2).

The very large increase in glyoxylic acid which occurred when seedlings were placed in nitrogen (Table III) indicates that the pathway of synthesis is not oxidative. It follows then that glycine (via oxidative deamination) glycolate, glycolaldehyde, and acetate are probably not sources of the carbon skeleton of glyoxylate, since their conversions involve oxidation. Under these conditions (anaerobiosis) neither pyruvate-1-C^{14}, pyruvate-2-C^{14}, nor acetate-2-C^{14} gave rise to glyoxylate, showing that the nitrogen-induced glyoxylate synthesis is not linked with carbohydrate or organic acid metabolism.

Glucose-C^{14} did not give rise to labelled glyoxylate although a number of intermediates of glycolysis and the tricarboxylic acid cycle became radioactive. Pyruvate-C^{14}, succinate-C^{14}, and acetate-C^{14} also gave rise to a variety of compounds including tricarboxylic acid cycle intermediates, but not to glyoxylate-C^{14}. These results show that isocitric acid and acetic acid are not important sources of glyoxylate. The extremely small amounts of glyoxylate-C^{14} formed from glycine-C^{14} in normal tissue suggests that this compound is a very unlikely precursor.

The feedings of solutions of various non-radioactive compounds to the roots of degrained seedlings growing in the dark (Table II) provide further information regarding the synthesis of glyoxylate. It was shown that neither glycine, glycolic acid, ribose, nor citric acid induced increases in the levels of glyoxylate. Since ribose may give rise to ribulose-5-phosphate and citric acid to isocitrate, these experiments further militate against a derivation from glycine, glycolaldehyde, glycolate, or isocitrate. The feeding of allantoin, on the other hand, did result in increased quantities of glyoxylate. The increase in glyoxylate and the decrease in allantoin in seedlings maintained in nitrogen (Table III) lends support to the idea that glyoxylate accumulates at the expense of allantoin under these conditions.

Little is known of the metabolism of allantoin and allantoic acid in plants although the work of Mothes and Engelbrecht (5, 11, 12) and Bollard (2) indicates that these compounds are of great importance in the nitrogen economy of many plants. Allantoin might be synthesized in the plant by the oxidation of purines, uric acid being an intermediate. Such a reaction sequence is well known in animals (4) and uricase, which catalyzes the breakdown of uric acid to allantoin, occurs widely in higher plants (14). Another mode of synthesis could be the condensation of two molecules of urea and one of glyoxylate, the reversal of the hydrolysis reaction. There is some evidence for the latter synthesis. A macerate of the tissue of *Agaricus campestris* has been found to catalyze the synthesis of allantoic acid from glyoxylate and urea (3). The leaves of *Platanus orientalis* are reported to contain no uricase but to have a high content of allantoin and allantoic acid (3), suggesting that these ureides do not arise from uric acid. It has also been reported quite recently, that carbon-14 from urea is incorporated more readily into Ehrlich-positive compounds such as allantoic acid, as well as into sugars, than into amino acids (6).

However, the results of the feedings of glyoxylate-C^{14} and glycine-C^{14} to wheat seedlings strongly suggest that glycine is the more immediate precursor of allantoin. The increased radioactivity in glyoxylate when glycine-C^{14} is fed and the plants maintained in nitrogen suggests that glycine is first incorporated into allantoin. The latter is hydrolyzed to give glyoxylate under these conditions. This interesting effect of nitrogen is not understood.

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THE EFFECT ON VARIATIONS IN DECAY OF MOISTURE CONTENT AND RATE OF GROWTH IN SUBALPINE SPRUCE[1]

D. E. ETHERIDGE[2]

Abstract

The moisture content in subalpine spruce was highest in overstory trees and lowest in understory trees, and it was higher in trees on a moist site than in trees of similar vigor classes on a dry site. Moisture contents of 10% of saturation for the more vigorous trees on the moist site and 7% of saturation for the less vigorous trees on the dry site represented a statistically significant difference which appeared to restrict the development of heartwood fungi to the wetter trees. A similar difference in the moisture content existed between the 1.5-ft. and the 20-ft. level of the trees, the occurrence of fungi being restricted to the wetter basal portion of the stems. In the laboratory, infection did not take place in test blocks at moisture contents lower than 8% of saturation. It is suggested that the threshold moisture content for infection in the trees occurs around the 7 to 8% saturation level. In the laboratory tests a difference in the moisture content of 3.4% of saturation in wood resulted in a statistically significant difference in the rate of decay by *Coniophora puteana* (Schum. ex Fr.) Karst., but differences in ring frequency and specific gravity in the test pieces corresponding to the differences between these properties in the living trees did not appreciably affect the decay rate. There was no evidence that variations in the distribution of decay-promoting or decay-retarding substances occurred in the trees from the two sites.

Introduction

The relationships between site and decay have received much study, but most investigators have dealt with the rating of decay losses by site quality classes or have attempted to assess hidden defect by the use of certain characteristics of the site and stand. According to Wagner and Davidson (22), who have recently reviewed much of the work published on this subject, considerably less has been done "to establish the fundamental reasons for the observed relationships, or to separate the influence of environment from others that may have some part in bringing about the decay status found in the particular stands under study". These authors feel that when the fundamental relationships have been sufficiently studied a better appreciation of the effects of site will be possible.

The tree characteristic most closely related to site and one which has received most attention in relation to decay is the rate of growth. However, the literature on this subject is conflicting, probably as a result of the absence of any satisfactory basis for analyzing and comparing the data. Evidence to show that decay is more rapid in fast-growing trees, or in trees on the better sites in Canada is given by McCallum (15), Basham (2), Davidson, Newell, and Cochrane (7), and Thomas and Thomas (21). On the other hand, Bier, Salisbury, and Waldie (3) found no consistent differences in the percentage of decay in fast- and slow-growing fir in the Upper Fraser region of British

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Columbia, and White (23) working in Ontario found a greater percentage of infected trees and larger volumes of decay in slow-growing white pine. These studies were based on the average condition in the residual trees in the different age classes, however, and make no provision for normal mortality in the stand which would result in the removal of infected trees at different ages. Thus, it is perhaps significant that the latter two studies were based on samples which contained almost twice as many trees in the slow-growing group. This might mean that mortality due to fungal infection in the different age classes, particularly in the older age classes, is much greater among the faster-growing trees. By the same token, conclusions based on the decay resistance of fast- and slow-growing mature and overmature stands determined by the use of site quality criteria, i.e., height growth at 50 or 100 years, should be interpreted with caution since it is generally recognized that good-quality sites, where growth is relatively rapid, possess fewer trees in the older age classes. This condition is due primarily to the dropping out of badly infected trees at an earlier age than occurs on poor sites which are more often characterized by a top-heavy age structure.

In a few instances attempts have been made to correlate the progress of decay in trees with the width of the annual growth rings and specific gravity of the wood, on the supposition that fungi penetrate more easily the less dense wood of the wide annual rings. Thus, Basham (2) found that the greatest depth of radial penetration (centripetally) in insect-killed balsam fir in Ontario tended to occur in those trees with wide rings in the outer sapwood. Passarge (16), studying this relationship in the Magdeburg Forest Region in Germany, found the maximum incidence of *Fomes pini* (Thore ex Fr.) Karst. attack on pine occurred on fertile soils with a high water table, and in trees with wide annual rings in the first 20 years of growth. According to Schulman (18), who has studied the longevity of several species of conifers in semiarid regions of the Western United States, extremely slow-growing overmature trees were relatively free of heart rot compared with fast-growing, favorably situated trees of the same age and species. This relationship between adverse site conditions, narrow annual rings, and a low incidence of rot appears to be corroborated by unpublished data obtained by the present author in a study of site factors in relation to the incidence of root- and butt-infections in subalpine spruce in Alberta. The relationships observed may be tabulated as follows:

	Dry site	Intermediate site	Moist site
Percentage of infected trees	10	22	52
Average width of annual rings			
Overstory trees	0.039 in.	0.047 in.	0.053 in.
Understory trees	0.027 in.	0.029 in.	0.032 in.

Although in this survey the incidence of infection has not been determined separately for overstory and understory trees, previous studies of decay in this species demonstrate a tendency for fewer infections to occur among the slower-growing trees of the understory (8).

Various attempts to discover the reasons for different rates of decay in slow-grown and fast-grown wood have been made by testing wood specimens in the laboratory under controlled conditions, but there is no conclusive evidence to indicate that the width of the annual rings or the specific gravity of the wood are the responsible factors (4, 20, 24). The possibility that certain substances or conditions occur in the heartwood of fast-growing trees that might act to promote the development of decay has not received adequate attention. The effect on decay of nitrogen compounds in the wood has been studied, *in vitro*, by a number of investigators (10, 14, 17), and while it appears that the rate of decay can be increased by adding certain of these compounds to test pieces of wood, it is not known whether the concentrations or the forms of nitrogen that were used compare with those in the living trees. Similarly, it has been shown that the moisture content of wood may confine the decay activity of fungi within a fairly narrow range (4, 19), but surprisingly little information is available on parallel relationships that might exist in the living tree. The only direct reference to such a relationship in living trees appears to be that by Henriksen and Jørgensen (13) in a study of factors to explain the higher incidence of attack by *Fomes annosus* (Fr.) Cooke in heavily thinned stands in Denmark. These investigators found that the moisture content in the trees in unthinned stands where the incidence of *F. annosus* was only 1%, was 23.9% of the oven-dry weight, whereas the moisture content in the most heavily thinned stands where the incidence of this fungus was 25%, was 31.2% of the oven-dry weight. The specific gravity of the wood was about 6% higher in the unthinned stands than in the heavily thinned stands, thus indicating that a faster growth rate as well as a higher moisture content was associated with the more heavily infected stands.

It therefore appears that fast-growing conifers are more prone to infection than slow-growing conifers but there are still differences of opinion on the nature of the decay-resisting properties of slow-grown wood. Although much valuable information has been obtained in laboratory studies on the relation of physical and chemical properties of wood to decay, it is difficult to assess the importance of these factors until more is known about how they compare with conditions in the living trees. It was the purpose of this investigation to consider these relationships by studying the relation between site and the characteristics of the heartwood of subalpine spruce, and, by controlled laboratory experiments, to examine the influence of these factors on decay.

Materials and Methods

Two suitable areas, representing a dry site and a moist site located in the Bow River Forest in the Rocky Mountain Forest Reserve in Alberta, were selected for the investigation on the basis of data obtained from previous surveys made in the region (9). The dry site, with characteristic plants of *Shepherdia canadensis* (L.) Nutt. and *Arctostaphylos uva ursi* (L.) Spreng., was located in a mixed stand of spruce (*Picea glauca* (Moench) Voss) and lodgepole pine, on the summit of a well-drained ridge, at an elevation of 4600–4800 ft. above sea level. The moist site, with characteristic plants of *Mertensia*

paniculata (Ait.) G. Don. and *Smilicina amplexicaulis* Nutt., was located about 4½ miles to the southeast in a pure stand of spruce, in a valley at an elevation of 4300–4500 ft. The estimated incidence of infected trees for the two stands was 0–5% and 60% respectively. The average gross volume for trees on the moist site was about twice that for trees of similar ages on the dry site (8).

Three overstory and three understory healthy trees on each of these sites were felled in July, 1954. The sites and the trees are briefly described in Table I.

TABLE I

SITE AND TREE DATA

Description of the site	Tree no.	Class of tree	Total ht. (ft.)	D.b.h. o.b. (in.)	Age at 1 ft. (yr.)
Dry site	1 DO	Overstory*	72.2	16.2	184
Aspect: flat, ridge top	4 DO	"	67.7	12.2	197
Slope: zero	5 DO	"	71.7	12.9	140
Elevation: 4600–4800 ft.	2 DU	Understory	47.1	5.9	110
Soil† pH: 5.5	3 DU	"	60.0	8.1	90
Soil texture: heavy	6 DU	"	41.3	6.9	102
Soil nitrogen: 0.136% (% dry wt.)					
Wet site	7 WO	Overstory	79.8	14.6	117
Aspect: south, valley bottom	8 WO	"	81.0	13.0	117
Slope: 16%	9 WO	"	75.4	11.7	115
Elevation: 4300–4500 ft.	10 WU	Understory	55.0	6.7	108
Soil pH: 6.1	11 WU	"	73.2	9.4	109
Soil nitrogen: 0.152% (% dry wt.)	12 WU	"	45.4	8.4	133

*Overstory trees are defined as dominant trees having a diameter at breast height outside bark (d.b.h.o.b.) greater than 11 in.

†Soil samples were taken from the "B" horizon: pH was estimated electrometrically from samples mixed with distilled water (1:1); nitrogen represents the total nitrogen of the sample.

The tree designations, DO, DU, WO, WU, refer to dry overstory, dry understory, wet overstory, and wet understory respectively.

Moisture determinations of the trees were made immediately after each tree was felled by taking samples, i.e., disks about 2 in. thick, from the stem at the butt level (at a height of from 1 to 2 ft. above ground level) and at distances of 20 and 40 ft. from the ground. Three blocks of heartwood, each about 2 in. square, were cut in sequence from the butt disk, the middle block of each group coming from the center of the disk. One block of heartwood was cut from each sample at the 20 and 40 ft. levels of the trees. After each sample block was cut, it was placed in an aluminum container and the lid quickly sealed with cellulose tape to prevent the loss of any water. In the laboratory, the containers together with the wood blocks were weighed, dried to a constant weight at 105° C., and reweighed. The difference between the initial weight and the final weight represented the amount of water in each sample. The moisture content was expressed as a percentage of the oven-dry weight (% o.d.w.) and as a percentage of the amount of water present at saturation

(% sat.). The latter was estimated approximately by a method which is based on the formula[3] given by Brown, Panshin, and Forsaith (5) for determining the moisture content in cell cavities of wood.

The specific gravity of the samples was calculated from their oven-dry weight and their green volume, i.e., when the moisture content was above the fiber saturation point, the samples were brought to the green volume by soaking in water for 24 hours; their volume was estimated to the nearest milliliter by immersing them in mercury in a graduated cylinder. The ring frequency was determined on specimens at the green volume by counting the average number of annual rings per inch on the end section of the samples.

The trees were also sampled by cutting sections, about 1 ft. in length, from the stem at the butt level, and at intervals of 10 ft. upward until the tree was less than 4 in. in diameter. The samples were marked as to their position in the tree before they were removed to the laboratory. In the laboratory, small samples $1\times1\times6$–7 in., were cut from the sapwood, the heartwood, and the center of each of the tree samples and these were maintained at the temperature and relative humidity of the laboratory until required for the experiments. For the decay experiments, nine blocks of wood measuring approximately $1\times1\times\frac{3}{4}$ in. were sawn in sequence from each sample and one end piece was selected for use in determining the moisture content. In the remaining eight pieces, holes were drilled halfway through one side of each block to receive the water amendment and a disk of wood inoculum. The nine blocks were then numbered and weighed. The oven-dry weight of each of the drilled blocks was calculated, assuming that they had the same moisture content as the selected end-block which had been oven-dried at 105° C. for 24 hours.

Decay chambers were wide-mouthed jars 5.5 cm. × 5.5 cm., with aluminum screw-top lids. The jars were thoroughly washed, rinsed in distilled water, and then sterilized in an oven for 2 hours at 160° C. The blocks were sterilized by steam or propylene oxide. When steam was used, the blocks were placed in the jars with the lids loosely screwed down. The jar assemblies were then steamed from the cold for $1\frac{1}{4}$ hours, $\frac{1}{2}$ hour of the time being at 100° C., and then allowed to cool slowly. This was repeated after 48 hours. The effect of the treatment on the initial moisture content in the blocks was determined by weighing control blocks after steaming. Propylene oxide was used in the way described by Hansen and Snyder (12). The blocks were placed on a perforated tray in a large desiccator into which propylene oxide was introduced at the rate of 1 ml. per liter capacity of the container. The blocks were left overnight, the gas was then removed with a suction pump, and the vessel flushed a number of times with filtered air. This treatment had no measurable effect on the initial moisture content of the blocks. The inocula used in these experiments were either plugs from agar plate cultures or pieces of infected wood. The agar plugs, about 5 mm. in diameter, were cut with a

[3]Percentage of saturation $=[(M-28)\ SG]/(1-0.93\ SG)$
where M = conventional moisture content; SG = the specific gravity of the wood based on the green volume assuming the fiber saturation point to be at 28% o.d.w., and the specific gravity of the dry wood 1.46.

cork borer from the plate cultures of the fungi grown on 2% malt extract agar. The wood inoculum was prepared in 250 ml. flasks by placing disks (about 22 mm. in diameter and 7 mm. thick) on a layer of spruce sawdust, about 3 g., to which was added 5% by weight of an accelerator recommended by Badcock (1) for increasing the rate of decay by wood-destroying fungi. Water was added at the rate of 170% of the oven-dry weight of the sawdust. After sterilizing, the flasks were inoculated with the test fungus and incubated for 3 months. The moisture content in the pieces of wood was kept at about 70 to 80% (o.d.w.) by periodically removing one of the disks from the flask, determining its moisture content, and then making the necessary adjustment to the remainder.

Before inoculating the test blocks their moisture content was adjusted by adding sterile distilled water from a hypodermic syringe calibrated to 0.1 ml. to the holes drilled in the side of each block. The amount of water that was added took into account the amount of water that was already in the blocks and the specific gravity of the wood. After making this initial adjustment to the moisture content, the blocks were inoculated with the test fungus. The lids were loosely screwed down on the jars and they were immediately placed in a saturated atmosphere in humidity chambers. For the purpose of adjusting the moisture, about midway through the incubation period, the blocks were removed from the jars in a room exposed for 1 hour to ultraviolet light from a germicidal lamp. After the disk of inoculum was removed from the blocks, they were weighed aseptically to the nearest 0.01 g. on a Jolly spring balance, the pan of which was contained in an inoculating hood. The water content of the blocks was then calculated; if it was above the level at which the test was being run, the blocks were allowed to lose water by being kept on the laboratory bench for a few days; if it was too low, the necessary amount of water was added from a hypodermic syringe. The decay activity was measured as the loss of weight during incubation as a percentage of the original oven-dry weight. The fungus used in the decay tests was an isolate of *Coniophora puteana* obtained from white spruce (*Picea glauca* (Moench) Voss) in Wasagaming, Manitoba.

Experimental

Site and Tree Relationships

The effect of site and dominance on growth and properties of the heartwood in subalpine spruce was investigated by sampling three overstory and three understory trees each from a moist and dry site. The results are given in Table II.

The influence of site and dominance on specific gravity and ring frequency was consistent in the four groups of trees, both the specific gravity and the number of annual rings per inch decreasing with increasing site moisture and with dominance. The specific gravity values show a regular increase from a low of 0.362 in the overstory trees on the moist site to a high of 0.408 in the understory trees on the dry site. Similarly, ring frequencies were lowest, viz., 12.5 annual rings per inch, in the overstory trees on the moist site, and

TABLE II

EFFECT OF SITE AND DOMINANCE ON SPECIFIC GRAVITY, RING FREQUENCY, AND THE MOISTURE CONTENT IN THE HEARTWOOD OF 12 HEALTHY SUBALPINE SPRUCE

Site and dominance	Tree no.	Origin of sample ht. in ft.	Specific gravity	Ring frequency	Moisture content	
					% o.d.w.	% sat.
Dry site overstory	1 DO	1.5 (P)[a]	—	—	59.1	—
		1.5	0.412	24.5	44.7	11.1
		1.5	0.340	36.0	51.6	11.8
		20.0	0.362	27.0	38.2	5.6
		40.0	0.363	19.0	36.0	4.4
		Average	0.369	26.6	45.9	8.2
	4 DO	1.5 (P)	—	—	42.8	—
		1.5	0.374	31.0	41.5	7.8
		1.5	0.409	31.5	33.7	3.7
		20.0	—	—	38.3	—
		40.0	0.378	19.0	37.8	4.5
		Average	0.387	27.1	38.8	5.3
	5 DO	1.5 (P)	—	—	47.6	—
		1.5	0.365	25.0	37.1	5.0
		1.5	0.384	24.0	48.3	12.1
		20.0	0.359	14.0	40.0	6.5
		40.0	0.462K[b]	14.0	36.4	6.8
		Average	0.392	19.0	41.9	7.6
		Class average	0.382	24.1	42.2	7.2
Dry site understory	2 DU	1.5 (P)	—	—	34.6	—
		1.5	0.450K	41.0	34.8	5.3
		1.5	0.442	42.0	35.2	5.4
		20.0	0.407	27.0	39.3	7.4
		Average	0.433	36.6	35.9	6.0
	3 DU	1.5 (P)	—	—	43.7	—
		1.5	0.404	27.0	51.4	15.2
		1.5	0.380	23.0	47.5	11.4
		20.0	0.413K	20.0	36.8	5.9
		40.0	0.426	13.0	35.5	5.3
		Average	0.406	20.7	42.9	7.5
	6 DU	1.5 (P)	—	—	39.3	—
		1.5	0.367	20.0	32.7	2.6
		1.5	0.433	39.0	39.3	8.2
		20.0	0.360	18.5	34.7	3.6
		Average	0.386	25.8	36.5	4.8
		Class average	0.408	27.0	38.8	7.0
		Site average	0.359	25.5	40.6	7.1
Moist site overstory	7 WO	1.5 (P)	—	—	40.0	—
		1.5	0.373	9.2	40.5	7.2
		1.5	0.346	12.8	62.4	16.8
		20.0	0.397	11.0	40.0	7.5
		40.0	—	—	38.8	9.4

TABLE II—*Concluded*

EFFECT OF SITE AND DOMINANCE ON SPECIFIC, RING FREQUENCY, AND THE MOISTURE CONTENT IN THE HEARTWOOD OF 12 HEALTHY SUBALPINE SPRUCE

Site and dominance	Tree no.	Origin of sample ht. in ft.	Specific gravity	Ring frequency	Moisture content	
					% o.d.w.	% sat.
Moist site overstory		Average	0.372	11.0	44.3	10.2
	8 WO	1.5 (P)	—	—	65.7	—
		1.5	0.358	10.5	62.2	18.8
		1.5	—	—	52.3	—
		20.0	0.338	11.0	43.2	7.5
		40.0	0.405K	9.5	35.9	5.1
		Average	0.367	10.3	51.8	10.5
	9 WO	1.5 (P)	—	—	59.3	—
		1.5	0.332	14.7	49.7	10.4
		1.5	0.350	17.5	55.0	14.1
		20.0	0.365	13.0	42.1	7.2
		40.0	0.364	16.0	39.4	6.2
		Average	0.353	15.3	49.1	9.5
		Class average	0.362	12.5	48.4	10.0
Moist site understory	10 WU	1.5 (P)	—	—	43.9	—
		1.5	0.329	15.0	36.0	3.7
		1.5	0.361	14.0	41.1	7.1
		20.0	0.357	15.5	37.4	5.0
		Average	0.349	14.8	39.6	5.3
	11 WU	1.5 (P)	—	—	55.2	—
		1.5	0.430K	14.0	47.7	19.5
		1.5	0.386K	15.0	47.4	11.7
		20.0	0.400K	10.5	40.0	7.2
		Average	0.402	13.1	47.6	10.8
	12 WU	1.5 (P)	—	—	54.6	—
		1.5	0.373	16.0	36.5	4.9
		1.5	0.385	22.0	53.1	15.1
		20.0	0.400K	16.0	36.6	5.5
		Average	0.386	18.0	45.2	8.5
		Class average	0.380	15.3	44.1	8.2
		Site average	0.369	18.8	46.5	9.2

[a](P) indicates samples were taken from the center of the heartwood and includes the pith.
[b]K indicates the presence of a knot in the sample.

NOTE: Significance of difference between means:

(i) Moist site vs. dry site
Moisture content (% o.d.w.), $P = .01$
Moisture content (% sat.), not significant
Specific gravity, $P = .02$
Ring frequency, $P = .001$

(ii) Moist site overstory vs. dry site understory
Moisture content { % o.d.w. $P = .01$ / % sat. $P = .02$

highest, viz., 27.0 rings per inch, in the understory trees on the dry site. This sequence held in the four groups of trees despite the much higher age of the three overstory trees on the dry site. A comparison of the means revealed statistically significant differences in specific gravity ($P = .02$), and highly significant differences in ring frequency ($P = .001$) between the trees on the two sites. It is evident that both specific gravity and ring frequency are related to the rate of growth of the trees, but of these properties, the number of rings per inch appears to be the better criterion of growth, and has the advantage by being the easier index of the two to measure.

Site and dominance show a marked influence on the amount of moisture present in the heartwood of the trees when moisture content is expressed either as a percentage of the dry weight or as a percentage of the amount of moisture present at saturation (Table II). The average moisture content in each of the four groups of trees appears to be quite clearly related to the rate of growth. The moisture content decreases from a maximum of 48.4% o.d.w. (10% sat.) in the faster-growing dominants on the moist site to a minimum of 38.8% o.d.w. (7.0% sat.) in the slow-growing understory trees on the dry site; differences are statistically significant (% o.d.w. $P = .01$; % sat. $P = .02$). When the mean moisture contents for the six trees on each of the two sites were compared, the difference was significant only when the moisture content was expressed on an oven-dry weight basis ($P = .01$). The fewer samples which were available for the percentage of saturation determinations probably explains this anomaly.

It is also evident in Table II that the moisture content values of the samples taken at 1.5 ft. are distinctly higher than of those taken at 20 ft. or higher in the trees. This condition appears to be characteristic of the pattern of distribution of moisture in the stems of the 12 trees and it does not appear to be connected with differences in the specific gravity of the wood.

Occurrence of Fungi in the Heartwood

To investigate the effect of site and dominance on the occurrence of heartwood fungi, 350 isolations were made on malt agar slants from disks of wood about 6 in. thick cut from sections of the stem at 1.5 ft. and at intervals of 10 ft. up the tree until a minimum diameter of 4 in. was reached. The results of this survey dealing with determinations of the fungi associated with the apparently healthy heartwood will be reported elsewhere. However, data on the distribution of the fungi in relation to variations in the moisture content in the stem and between the trees are sufficiently pertinent to be summarized here.

It is especially noteworthy that no fungi were present in samples where the moisture content was less than 5% of saturation at the time of felling, whereas 36.3% of the samples in the 5 to 10% saturation range, and 62.5% of those in the 10 to 15% range, were infected by various species of fungi. The percentage of samples that yielded fungi from fast- and slow-growing trees on the wet and dry sites was, respectively, 13.3, 11.1, 7.0, and 0.0. It is of interest that no fungi were isolated from the three understory trees on the dry site where

the average moisture content was only 7.0% of saturation. Invariably fungi were confined to the wetter basal portion of the trees, none being isolated from samples higher than 20 ft. from the ground.

Laboratory Studies of Factors Influencing the Rate of Decay

Preliminary trials had shown that wood test blocks having a specific gravity of 0.390 decayed about twice as rapidly as those having a specific gravity of 0.335 despite near uniform moisture contents of 42.6 and 45.1% o.d.w. in the samples, a difference of only 2.5%. Because of the greater dry weight of the dense samples, it was obvious that they held much more water than the difference in these values suggested. Therefore, using the formula given earlier,[3] the moisture in the samples was recalculated as a percentage of the water present at saturation. Values of 6.6% for the less dense, and 10.4% for the dense samples were obtained which indicated that nearly twice as much of the cell cavity had been occupied by water in the more rapidly decayed wood. Consequently, in the following experiments where it was necessary to compare the rate of decay in wood of different densities, the amount of moisture was expressed as percentage of saturation. In the preliminary work the initial moisture content of the blocks was maintained during the test period by making adjustments to the moisture level on the basis of changes occurring in the control blocks which were sampled periodically. This method, however, required a large number of additional blocks since samples of different densities and of different treatments gained or lost water at different rates during incubation. As it was difficult to handle such a large number of control blocks, this method was abandoned in favor of the method described earlier which entailed weighing each block aseptically midway through the incubation period and then adjusting the moisture to the required level.

By employing these techniques, the effect on decay of site, dominance class, moisture, and properties of the heartwood was investigated. One hundred and ninety-two specimens from two positions in each of the 12 trees were prepared as already described. A statistical design employing the split-plot system of analysis was used. The principal object of the experiment was to discover whether the site, dominance class, or position in the tree, i.e., butt and trunk, from which the specimens of heartwood came had any effect on the rate of decay by *Coniophora puteana*. Three other variables were conveniently introduced and added considerably to the value of the experiment. These were: (*a*) moisture content, (*b*) method of sterilization, i.e., steam and propylene oxide, and (*c*) two different types of inocula. In addition, any effect on decay by such variables as ring frequency and specific gravity could be investigated by evaluating differences between the decay rates of the four vigor classes. Eight test blocks were used from each of the two positions in the 12 trees. Of these, one series of four was inoculated with pieces of infected wood, and another series with agar plugs. Two replicates of each series were sterilized in propylene oxide gas while the other two replicates were sterilized in steam as described earlier. One replicate series of each sterilization

treatment was kept at different moisture levels. At the end of 3 months, the series inoculated with agar plugs, a total of 96 test blocks, had decayed very little compared with the series inoculated with disks of infected wood. These, therefore, were left for another month and the results were analyzed separately. The results for the series inoculated with wood disks are set out in Table III. An analysis of variance was made with these data, the scheme consisting of data from 12 trees, each tree furnishing data from samples taken at the butt (1.5 ft.) and the trunk (20 ft.). Each sample from each position consisted of four test blocks which included all combinations (2×2) of the sterilization and moisture treatments. There were, therefore, in the experiment, three sections dealing with comparisons between the 12 trees, 24 positions, and 96 test blocks, entailing in the analysis of variance three separate estimates of error, each one applicable to its own particular treatment comparisons. Accordingly, the variances of the components in each of these three sections were tested against the particular error variance calculated for the section. The analysis of variance is set out in Table IV. When the items were tested by the use of variance ratio tables, only moisture had any significant effect on the rate of decay, having a probability of less than .05. The chief results of the experiment are given below under appropriate headings.

TABLE III

PERCENTAGE LOSS IN WEIGHT OF BLOCKS OF WOOD OF SUBALPINE SPRUCE DECAYED BY *Coniophora puteana*, AFTER 3 MONTHS

Tree no.	Position in tree	Method of sterilizing blocks: Propylene oxide M_1*	Propylene oxide M_2	Steam M_1	Steam M_2	Total M_1	Total M_2	Grand total
Dry site overstory								
1 DO	Butt	13.2	15.1	10.8	12.7	24.0	27.8	51.8
	Trunk	12.2	11.9	17.9	12.1	30.1	24.0	54.1
	Total	25.4	27.0	28.7	24.8	54.1	51.8	105.9
4 DO	Butt	10.7	9.1	13.1	8.4	23.8	17.5	41.3
	Trunk	10.9	10.9	9.5	12.1	20.4	23.0	43.4
	Total	21.6	20.0	22.6	20.5	44.2	40.5	84.7
5 DO	Butt	11.8	10.5	10.8	7.9	22.6	18.4	41.0
	Trunk	12.9	11.0	8.0	9.6	20.9	20.6	41.5
	Total	24.7	21.5	18.8	17.5	43.5	39.0	82.5
Position totals	Butt	35.7	34.7	34.7	29.0	70.4	63.7	134.1
	Trunk	36.0	33.8	35.4	33.8	71.4	67.6	139.0
Class totals		71.7	68.5	70.1	62.8	141.8	131.3	273.1
Dry site understory								
2 DU	Butt	14.7	11.8	14.1	13.4	28.8	25.2	54.0
	Trunk	5.7	8.1	15.0	14.2	20.0	22.3	42.3
	Total	19.7	19.9	29.1	27.6	48.8	47.5	96.3

TABLE III—*Concluded*

PERCENTAGE LOSS IN WEIGHT OF BLOCKS OF WOOD OF SUBALPINE SPRUCE DECAYED BY *Coniophora puteana*, AFTER 3 MONTHS

Tree	Position in tree	Method of sterilizing blocks: Propylene oxide M'*	Propylene oxide M_2	Steam M'	Steam M_2	Total M'	Total M_2	Grand total
Dry site understory								
3 DU	Butt	9.4	10.0	9.3	7.1	18.7	17.1	35.8
	Trunk	12.0	13.2	18.3	14.1	30.3	27.3	57.6
	Total	21.4	23.2	27.6	21.2	49.0	44.4	93.4
6 DU	Butt	12.1	10.5	9.5	8.7	21.6	19.2	40.8
	Trunk	9.9	12.3	10.4	9.8	20.3	22.1	42.4
	Total	22.0	22.8	19.9	18.5	41.9	41.3	83.2
Position totals	Butt	36.2	32.3	32.9	29.2	69.1	61.5	130.6
	Trunk	26.9	33.6	43.7	38.1	70.6	71.7	142.3
Class totals		63.1	65.9	76.6	67.3	139.7	133.2	272.9
Total for site		134.8	134.4	146.7	130.1	281.5	264.5	546.0
Wet site overstory								
7 WO	Butt	14.5	15.0	14.3	13.1	28.8	28.1	56.9
	Trunk	14.8	11.8	15.0	14.8	29.8	26.6	56.4
	Total	29.3	26.8	29.3	27.9	58.6	54.7	113.3
8 WO	Butt	13.0	8.0	13.4	13.4	26.4	21.4	47.8
	Trunk	9.1	9.9	11.9	10.8	21.0	20.7	41.7
	Total	22.1	17.9	25.3	24.2	47.4	42.1	89.5
9 WO	Butt	11.8	12.9	11.4	10.8	23.2	23.7	46.9
	Trunk	10.5	10.6	9.8	11.5	20.3	22.1	42.4
	Total	22.3	23.5	21.2	22.3	43.5	45.8	89.3
Position totals	Butt	39.3	35.9	39.1	37.3	78.4	73.2	151.6
	Trunk	34.4	32.3	36.7	37.1	71.1	69.4	140.5
Class totals		73.7	68.2	75.8	74.4	149.5	142.6	292.1
Wet site understory								
10 WU	Butt	10.8	10.4	11.4	12.2	22.2	22.6	44.8
	Trunk	21.2	12.5	13.6	11.4	34.8	23.9	58.7
	Total	32.0	22.9	25.0	23.6	57.0	46.5	103.5
11 WU	Butt	13.8	13.0	15.5	14.4	29.3	27.4	56.7
	Trunk	10.4	10.0	10.6	9.0	21.0	19.0	40.0
	Total	24.2	23.0	26.1	23.4	50.3	46.4	96.7
12 WU	Butt	11.6	7.2	6.4	7.8	18.0	15.0	33.0
	Trunk	12.1	8.8	10.3	11.3	22.4	20.1	42.5
	Total	23.7	16.0	16.7	19.1	40.4	35.1	75.5
Position totals	Butt	36.2	30.6	33.3	34.4	69.5	65.0	134.5
	Trunk	43.7	31.3	34.5	31.7	78.2	63.1	141.2
Class totals		79.9	61.9	67.8	66.1	147.7	128.0	275.7
Total for site		153.6	130.1	143.6	140.5	297.2	270.6	567.8

*(M_1) Moisture content at 60% of the dry weight.
(M_2) Moisture content at 14% of saturation.

TABLE IV

ANALYSIS OF VARIANCE OF LOSS IN WEIGHT OF BLOCKS OF WOOD OF SUBALPINE SPRUCE DECAYED BY *Coniophora puteana*, AFTER 3 MONTHS

Factor	Sum of squares	Degrees of freedom	Variance	Variance ratio
Wet site vs. dry site	4.95	1	4.95	3.90
Overstory vs. understory	2.86	1	2.86	6.75
Site vs. dominance class interaction	2.76	1	2.76	7.00
Error (i)	154.48	8	19.31	
Total (i)	165.05	11		
Butt vs. trunk	1.55	1	1.55	11.50
Butt vs. trunk interaction	12.14	3	4.05	4.40
Error (ii)	142.70	8	17.83	
Total (ii)	321.44	23		
Treatments				
Sterilization, propylene oxide vs. steam	0.66	1	0.66	6.13
Moisture content 60% dry wt. vs. 14% sat.	19.80	1	19.80*	4.80*
Sterilization vs. moisture interactions	0.79	1	0.79	5.12
Remaining interactions	94.51	21	4.50	1.11
Error (iii)	194.58	48	4.05	
Total (iii)	631.78	95		

*Significant at 5% point.

Moisture

It has already been pointed out that when the moisture content was expressed as a percentage of the dry weight, an erroneous picture was obtained of the amount of water present in samples of wood of different densities. Therefore, comparisons were made after first converting moisture content values (% o.d.w.) to percentage of saturation by the formula.[3] Two different moisture treatments were tested which gave two levels of moisture in the experiment. To one series (called M_1), an amount of water equal to 60% of the dry weight was added which ignored the differences in densities of the blocks, while to another series (M_2), an amount of water was added which gave a uniform moisture content of 14% of saturation. The relation between the moisture content values in the two series is expressed by the formula:

$$60\% \text{ o.d.w.} = 14/0.311 + 14.98$$
(M_1 series)

where 14.98 is a constant, 14 is the percentage of saturation value chosen for the M_2 series, and 0.311 is the specific gravity value for the least dense of the test blocks to be tested, i.e., samples from overstory trees on the moist site. The amount of water that was added to the test blocks with specific gravities greater than 0.311 was determined by substituting the appropriate S.G. value in the formula. Thus, in the M_1 series the initial moisture content of the blocks increased from 14 to 19.1% of saturation over a range of specific gravities of from 0.311 to 0.424, whereas, in the M_2 series, the moisture content was kept constant at 14% of saturation over this range by simply adding proportionally less water as the specific gravity of the blocks increased.

Table V, which sets out the mean moisture contents (% sat.) corresponding to the decay values given in Table III, illustrates the results that were obtained. The average moisture content values for the M_2 series were relatively

TABLE V

MEAN MOISTURE CONTENT (% SATURATION), DURING INCUBATION, OF BLOCKS OF WOOD OF SUBALPINE SPRUCE DECAYED BY *Coniophora puteana*

Tree no.	Position in tree	Moisture treatment* M_1 Prop. Oxide	M_1 Steam	M_1 Av.	M_2 Prop. oxide	M_2 Steam	M_2 Av.
Dry site overstory							
1 DO	Butt	20.2	22.0	21.1	16.6	17.8	17.2
	Trunk	17.8	18.7	18.2	15.0	16.4	15.7
4 DO	Butt	21.1	20.5	20.8	15.8	13.8	14.8
	Trunk	18.8	15.8	17.3	16.2	14.3	15.2
5 DO	Butt	23.0	21.3	22.1	15.2	13.9	14.5
	Trunk	20.0	16.8	18.4	17.8	15.6	16.7
Class total or average		120.9	115.1	19.6	96.6	91.8	15.8
Dry site understory							
2 DU	Butt	25.9	29.4	27.6	18.2	21.6	19.9
	Trunk	20.6	28.0	24.3	16.9	19.4	18.1
3 DU	Butt	25.8	21.8	23.8	17.3	16.2	16.7
	Trunk	22.2	21.4	21.8	17.2	16.6	16.9
6 DU	Butt	22.7	20.4	21.5	16.7	13.5	15.1
	Trunk	22.6	21.6	22.1	19.7	17.3	18.5
Class total or average		139.8	142.6	23.5	108.0	104.6	17.5
Dry site total or average		260.7	257.7	21.6	204.6	196.4	16.6
Wet site overstory							
7 WO	Butt	15.4	17.2	16.3	15.4	19.7	17.5
	Trunk	19.0	19.5	19.2	19.1	16.9	18.0
8 WO	Butt	17.0	16.2	16.6	14.9	19.1	17.0
	Trunk	17.3	16.9	17.1	16.3	15.0	15.6
9 WO	Butt	19.4	19.1	19.2	16.2	16.2	16.2
	Trunk	18.5	17.1	17.3	18.7	16.2	17.4
Class total or average		106.6	106.0	17.7	100.6	103.1	16.9
Wet site understory							
10 WU	Butt	19.5	20.0	19.7	17.3	18.9	18.1
	Trunk	23.0	18.5	20.7	16.5	17.8	17.1
11 WU	Butt	22.0	20.2	21.1	18.8	18.5	18.6
	Trunk	20.7	19.6	20.1	15.9	15.2	15.5
12 WU	Butt	19.1	18.3	18.7	19.1	16.1	17.6
	Trunk	19.1	18.7	18.9	15.8	14.3	15.0
Class total or average		123.4	115.3	19.9	103.4	100.8	17.0
Wet site total or average		230.0	221.3	18.7	204.0	203.9	16.9

*(M_1) Moisture content at 60% of the dry weight.
(M_2) Moisture content at 14% of saturation.

uniform at 16.9 and 16.6% of saturation respectively in samples from the two sites, despite the wide spread in densities in these samples. In contrast, in the M_1 series where no attempt was made to adjust for the different densities, the average moisture content value for the denser dry-site samples was 21.6% and that for the less dense samples from the moist site, 18.7%. The average moisture content in the M_1 series was 20.2% and in the M_2 series, 16.8%, representing a difference between the moisture treatments of 3.4% of saturation. The percentage loss in weight due to decay in series M_1 and M_2 was 12.05 and 11.30 respectively. Thus, a difference of 3.4% of saturation between the treatments resulted in a statistically significant difference of 0.75% in decay (Table VI). It is noteworthy that differences in the moisture content smaller than 3.4%, viz., 1.2% between the two sites, 2.0% between the two dominance classes, and 0.7% between the two positions, did not result in significant differences in the rate of decay by *C. puteana*.

When the data were arranged into specific gravity and ring frequency classes, without exception, the amount of decay increased regularly with increasing moisture content, but there was no apparent relation between decay and the structural properties of the wood.

TABLE VI

CHARACTERISTICS OF THE SPECIMENS OF SUBALPINE SPRUCE DECAYED BY *Coniophora puteana* IN RELATION TO SITE, DOMINANCE CLASS, POSITION, AND TREATMENT FACTORS AS TESTED BY ANALYSIS OF VARIANCE

	Average*			
Factor analyzed	Percentage saturation	Loss in weight (% dry wt.)	Ring frequency	Specific gravity
Wet site	17.8	11.9	18.0	0.344
Dry site	19.0	11.3	28.2	0.372
Difference	1.2	0.6	10.2	0.028
Overstory	17.4	11.7	19.1	0.339
Understory	19.4	11.5	27.1	0.377
Difference	2.0	0.2	8.0	0.038
Butt	18.8	11.4	26.4	0.366
Trunk	18.1	11.7	19.8	0.350
Difference	0.7	0.3	6.6	0.016
Moisture 60% o.d.w.	20.2	12.05	Samples from the same piece of wood	
Moisture 14% sat.	16.8	11.30		
Difference	3.4	0.75†	None	None

*Each value is based on the following number of observations:
Percentage of saturation 48
Loss in weight 48
Specific gravity 12
Ring frequency 12
†The difference is significant at the 5% point.

Specific Gravity and Ring Frequency

From the foregoing analysis there was no consistent evidence of a relationship between ring frequency and decay, or between specific gravity and decay, but there was a tendency for high moisture contents to be associated with the denser wood samples. Possibly this condition may have obscured the effect of any decay-resisting properties that were associated with the dense wood. This was investigated by selecting samples whose moisture contents were in the relatively narrow range of 17.5 to 19.5% of saturation and then determining the average decay values for ascending ring frequency classes (Table VII), but there was no evidence of a relation between ring frequency and decay with the moisture content thus controlled.

TABLE VII

RELATION BETWEEN RING FREQUENCY AND LOSS IN WEIGHT IN SPECIMENS OF SUBALPINE SPRUCE DECAYED BY *Coniophora puteana*, MOISTURE CONTENT APPROXIMATELY UNIFORM

Ring frequency class	No. of samples	Average ring frequency	Average moisture content (% sat.)	Average loss in weight (% dry wt.)
8 – 12	3	10.8	18.2	14.1
12 – 16	1	13.0	18.9	11.2
16 – 20	1	20.5	18.4	10.4
20 – 24	3	23.3	18.5	10.6
24 – 28	2	26.2	18.3	13.0
28 – 32	0	—	—	—
32 – 36	2	36.0	18.3	12.4
36 – 40	1	38.0	18.1	11.3

Site and Dominance

Site and dominance class had no statistically significant effect on decay when the decay rates for 48 test blocks in each of these categories were compared and tested by analysis of variance (Tables IV and VI). There was a tendency, however, for decay to be greater in the samples from the moist-site trees than in those from the dry-site trees. Similarly, decay was greater in the samples from the overstory than in those from the understory. It is noteworthy that slightly lower moisture values were recorded for the two categories where greater decay losses occurred. However, because a close relationship existed between the physical characteristics of the trees, i.e., ring frequency and specific gravity, and site and dominance, it was not possible to separate the effect on decay of these properties from others which may also characterize the heartwood of the different trees.

Position in the Stem

A comparison between 48 samples of heartwood from the butt (at 1.5 ft. from the ground) and an equal number of samples from the trunk (at 20 ft. from the ground) revealed differences of only 0.3% in the average loss in weight

due to decay (Tables IV and VI). The samples from the butt were characterized by higher average values for ring frequency and specific gravity, and slightly lower moisture contents than the corresponding samples from the trunk position.

Method of Sterilization

Since it was possible that certain natural constituents of the heartwood, viz., resins, might be affected by steaming the test blocks during the sterilization process, it was necessary to investigate this factor. Consequently, one series of 48 test blocks was sterilized by steaming and another series which served as controls was sterilized over propylene oxide gas in the manner already described. The two methods were compared and tested by analysis of variance (Tables III and IV). The average loss by decay in the steam-sterilized blocks was 11.85% of oven-dry weight compared with 11.50% in the blocks sterilized with propylene oxide, but this difference was not statistically significant. The experiment demonstrated, therefore, that decay-resisting resins or other volatile substances, which might be removed or changed by steaming, were unimportant in preventing decay by *C. puteana*. Both methods were equally effective as sterilizing agents.

Source of Inoculum

As already stated, the total of 192 test blocks was divided into two series. The blocks of one series were inoculated with disks of infected wood, one disk occupying each cavity. Those of the other series were inoculated with plugs of agar from plate cultures; two plugs were placed diametrically opposite at the bottom of each cavity. The rate of decay in the blocks inoculated with wood disks was obviously greater at the end of 3 months than in the blocks inoculated with agar plugs; therefore, the latter were left for another month. In the agar-inoculated blocks the average decay loss after 4 months was 5.9% of the dry weight, whereas, in the wood-inoculated blocks, the average decay loss was twice as great after only 3 months; infection was well established after 3 weeks in the latter and after 6 weeks in the former.

Because the two treatments differed so greatly in the effect on decay, the data were examined to discover the reason for the appreciably slower decay of the agar-inoculated blocks. A comparison was made of the moisture values for 128 successfully infected test blocks representing four trees and two sites from each of the two inoculation treatments. Each of the four dominance classes in the agar-inoculated series had lower moisture values than was the case for the wood-inoculated series but the differences were not large enough to account for the large differences in decay. For example, when the data for each of the two series were arranged into moisture classes, and the average values for moisture were plotted against decay values, two distinct curves were obtained (Fig. 1) which indicated that moisture was not the determining factor. Differences in decay rates, therefore, could be attributed either to differences in size or nutritional value of the inoculum, i.e., the inoculum potential (Garrett (11)). It is unlikely that a disk of wood contained more of

any growth factor than the two agar plugs, but probably contained more water. However, in the saturated atmosphere of the decay chambers, the effect of the water content of the inoculum was probably very slight. The evidence, although inconclusive, suggests therefore that it was the larger mass of wood inoculum and the larger area of contact that was responsible for the increased rate of decay. Since 12 test blocks representing two trees in the agar-inoculated series were contaminated or infection failed to take by the end of 4 months, the data were not tested by analysis of variance but comparisons of decay losses similar to those between items in the wood-inoculated series were obtained.

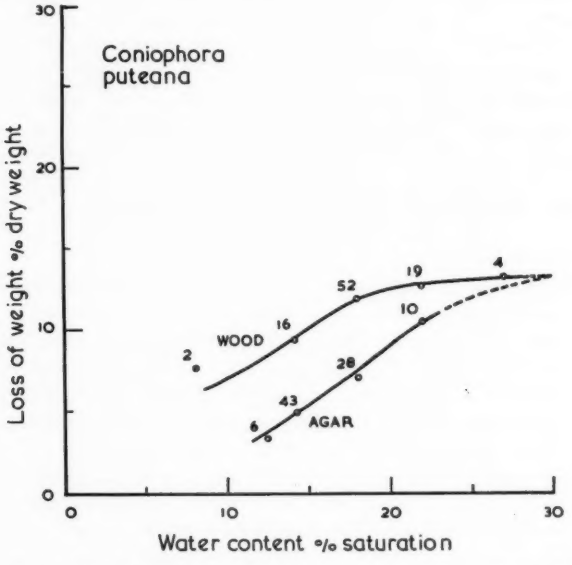

FIG. 1. Curves showing the relationship between the decaying activity of *Coniophora puteana*, the moisture content of the substratum, and the method of inoculating the test blocks. Wood-inoculum series at the end of 3 months, agar-inoculum series at the end of 4 months.

Discussion

One of the results of this investigation has been to demonstrate a connection between site moisture conditions, growth and vigor of the trees, and the moisture content in the heartwood of subalpine spruce. These results are similar to those obtained by Chalk and Bigg (6) for Sitka spruce in Britain, who found that the moisture content in the sapwood tended to be highest in the more vigorous trees on the moist sites. Since the conclusions in both these investigations have been based on comparisons when the moisture content was expressed as a percentage of the moisture held by the wood at saturation, it is unlikely that the results can be explained by differences in the specific

gravity of the samples. There is evidence that the distinctly wetter heartwood in trees on the moist site and in overstory trees is directly influenced by the amount of available water, not by the properties of wood associated with the faster rate of growth. For instance, tree No. 12 WU on the moist site, and tree No. 5 DO on the dry site have similar densities and ring widths but differ only in their moisture contents; similarly, an overstory tree, viz., No. 9 WO on the moist site, had a distinctly higher moisture content than an understory tree, viz., No. 10 WU growing on the same site, in spite of otherwise similar characteristics of the heartwood. Moreover, there was a sharp drop in the moisture content in all the trees at the 20-ft. level of the stem and a further drop at the 40-ft. level which bore no apparent relation to the specific gravity of the wood.

It is shown here that while a difference in the moisture content of only 3% of saturation existed between the fast-grown dominants on the moist site and the slow-growing understory trees on the dry site, this small difference was statistically significant and it had a marked effect on the occurrence of fungi in the trees. However, the point of interest is that the lower moisture level characterizing the heartwood of the dry-site trees was sufficiently critical to prevent infection by the fungi. For example, 13.3% of the samples from the moist-site trees having an average moisture content of 10% saturation yielded fungi, whereas no fungi were isolated from the dry-site samples which had moisture contents averaging 7%. A similar pattern in the distribution of moisture appears to explain the absence of fungi above the 20-ft. level of the trees since average moisture content values in the butt and upper portions of the stem were, respectively, 9.8 and 6.0%. It is therefore probable that the threshold moisture content for infection in the trees lies somewhere around 7% of saturation. This is borne out by laboratory experiments which have indicated that infection fails to take place in test blocks with moisture contents lower than 8% saturation (Fig. 1).

A significant effect on decay induced by small changes in the moisture content of wood has been clearly demonstrated by the laboratory tests. Results of this study have shown that a difference in the moisture content of as little as 3.4% of saturation has a critical effect on the rate of decay by *C. puteana*. It has also been seen that when moisture is controlled the differences in ring frequency and specific gravity, which represented differences between these properties in the living trees, did not result in significant differences in decay by this fungus. Neither is there any evidence that decay is influenced by the origin of the samples. This appears to exclude the possibility that variations in the distribution of decay-promoting or decay-retarding substances occur in the trees.

Although only one fungus was used in the tests, there is some evidence from collateral studies that other species of wood-destroying fungi occurring on subalpine spruce have similar moisture requirements. Tests with seven different species of wood-destroying fungi have shown that moisture contents of from 5 to 10% of saturation are needed before any appreciable decay can occur.

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NOTES

PENIOPHORA PHLEBIOIDES JACKSON AND DEARDEN, A SAPWOOD DETERIORATING FUNGUS ON EASTERN WHITE PINE[1]

S. N. LINZON[2]

In 1949 a study of the effects of smelter fumes on forest growth in the Sudbury and surrounding districts of Ontario (2) was begun by the Forest Pathology Laboratory, Maple, Ontario. In this study a continuous record of the annual mortality of white pine (*Pinus strobus* L.) located in 45 permanent sample plots was maintained. In addition, each year a number of living white pines were felled in the several study areas and left lying on the ground in log lengths. It was anticipated that these dead trees would provide data on the rates of deterioration of both standing and fallen white pines, and a record of the succession of fungi which may infect and cause progressive deterioration of the wood.

In 1953 a number of felled trees, and in 1954 a number of dead standing trees, were selected for preliminary examination. The trees were dissected and the pattern of deterioration was recorded. Isolations were made from stained and decayed sapwood, with routine identification of fungi in culture being undertaken at Maple. Those that could not be determined in this way were identified by Dr. M. K. Nobles, Botany and Plant Pathology Division, Ottawa. Numerous Fungi Imperfecti were isolated from the sapwood. Several Basidiomycetes also were isolated and are here listed in the order of frequency of occurrence.

From dead standing white pine: *Polyporus abietinus* Dicks. ex Fr., *Polyporus anceps* Peck, *Lenzites saepiaria* Wulf. ex Fr., *Corticium galactinum* (Fr.) Burt, *Peniophora gigantea* (Fr.) Massee, *Poria subacida* (Peck) Sacc., *Coniophora puteana* (Schum. ex Fries) Karst., *Corticium laeve* Pers., and *Trechispora coronifera* (Hahn & Litsch.).[3]

From fallen white pines: *Peniophora gigantea* (Fr.) Massee, *Polyporus anceps* Peck, *Peniophora phlebioides* Jackson and Dearden, *Lenzites saepiaria* Wulf. ex Fries, *Corticium galactinum* (Fr.) Burt, *Polyporus abietinus* Dicks. ex Fr., and *Trechispora brinkmanni* (Bres.) Rog. & Jacks.

Peniophora phlebioides has not previously been recorded as a saprophyte of eastern white pine. The fungus was described for the first time by Jackson and Dearden (1) in 1949. Identification of *P. phlebioides* in culture was made by Dr. Nobles through comparison of fruit bodies in culture with collections of the sporophore from Douglas fir made in British Columbia. The single known collection of the sporophore in Ontario was made by the author in 1955.

[1]Contribution No. 420 from the Forest Biology Division.
[2]Laboratory of Forest Pathology, Maple, Ontario.
[3]Tentatively identified in the absence of comparative material. Dr. M. K. Nobles reports, however, that the fungus in culture agrees well with the description of *T. coronifera*.

P. phlebioides was isolated in 1953 from four of 12 white pines examined in this preliminary study. Isolations were obtained from the sapwood of logs excised from the midportion of trees that had been dead for either 2 or 3 years. The affected logs were located in two townships, Lewis and Clement, Ontario, which are approximately 110 miles apart. In each case logs were exposed on rocky slopes and their location suggests that the development of this fungus may be related to specific temperature and moisture conditions.

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